



# The Journal of Biological Chemistry

Volume 5  
1908-1909

*Printed by arrangement with the American Society of Biological Chemists, Inc.*

JOHNSON REPRINT CORPORATION

New York, New York



# THE JOURNAL

OF

# BIOLOGICAL CHEMISTRY

EDITED BY

J. J. ABEL  
Baltimore

and

C. A. HERTER  
New York

Assistant Editor, A. N. RICHARDS, Chicago

WITH THE COLLABORATION OF

R. H. CHITTENDEN, New Haven, Conn.

OTTO FOLIN, Waverley, Mass.

WILLIAM J. GIES, New York.

REID HUNT, Washington, D. C.

WALTER JONES, Baltimore, Md.

J. H. KASTLE, Washington, D. C.

WALDEMAR KOCH, Chicago, Ill.

P. A. LEVENE, New York.

JACQUES LOEB, Berkeley, Cal.

GRAHAM LUSK, New York.

A. B. MACALLUM, Toronto, Canada.

J. J. R. MACLEOD, Cleveland, Ohio.

A. P. MATHEWS, Chicago, Ill.

L. B. MENDEL, New Haven, Conn.

F. G. NOVY, Ann Arbor, Mich.

W. R. ORNDORFF, Ithaca, N. Y.

THOMAS B. OSBORNE, New Haven, Conn.

FRANZ PFAFF, Boston, Mass.

A. E. TAYLOR, Berkeley, Cal.

V. C. VAUGHAN, Ann Arbor, Mich.

ALFRED J. WAKEMAN, New York.

HENRY L. WHEELER, New Haven, Conn.

VOLUME V

BALTIMORE

1908-1909



COPYRIGHT 1908 AND 1909  
BY  
THE JOURNAL OF BIOLOGICAL CHEMISTRY.

WILLIAMS & WILKINS COMPANY PRESS  
BALTIMORE, MD.

## CONTENTS OF VOLUME V.

WALTER JONES: On the identity of the nucleic acids of the thymus, spleen and pancreas . . . . .	1
P. G. HEINEMANN: Note on the concentration of diphtheria toxin . . . . .	27
T. BRAILSFORD ROBERTSON and C. L. A. SCHMIDT: On the part played by the alkali in the hydrolysis of proteins by trypsin . . . . .	31
TREAT B. JOHNSON and SAMUEL H. CLAPP: IX. Researches on pyrimidins: Synthesis of some nitrogen-alkyl derivatives of cytosin, thymine and uracil . . . . .	49
MATTHEW STEEL and WILLIAM J. GIES: Some notes on the efficiency of the Folin method for the quantitative determination of urinary ammonia . . . . .	71
MATTHEW STEEL: A study of the influence of magnesium sulfate on metabolism . . . . .	85
JOHN F. LYMAN: A note on the chemistry of the muscle and liver of reptiles . . . . .	125
H. GIDEON WELLS: The chemistry of the liver in chloroform necrosis (delayed chloroform poisoning) . . . . .	129
T. BRAILSFORD ROBERTSON: On the influence of temperature upon the solubility of casein in alkaline solutions . . . . .	147
T. BRAILSFORD ROBERTSON: Note on the applicability of the laws of amphoteric electrolytes to serum globulin . . . . .	155
TREAT B. JOHNSON and SAMUEL H. CLAPP: X. Researches on pyrimidins: The action of diazobenzene sulphuric acid on thymine, uracil and cytosin . . . . .	163
H. D. DAKIN: Further studies of the mode of oxidation of phenyl derivatives of fatty acids in the animal organism . . . . .	173
THOMAS B. OSBORNE and FREDERICK W. HEYL: Hydrolysis of vicilin from the pea ( <i>Pisum sativum</i> ) . . . . .	187
THOMAS B. OSBORNE and FREDERICK W. HEYL: Hydrolysis of legumelin from the pea ( <i>Pisum sativum</i> ) . . . . .	19

O. F. BLACK: The detection and quantitative determination of $\beta$ -oxybutyric acid in the urine . . . . .	207
PHILIP A. SHAFFER: A method for the quantitative determination of $\beta$ -oxybutyric acid in the urine . . . . .	211
FRANK P. UNDERHILL and TADASU SAIKI: The influence of complete thyroidectomy and of thyroid feeding upon certain phases of intermediary metabolism . . . . .	225
C. L. ALSBERG and E. D. CLARK: On a globulin from the egg yolk of the spiny dog-fish, <i>Squalus Acanthias</i> . . . . .	243
RACHEL H. COLWELL and H. C. SHERMAN: Chemical evidence of peptonization in raw and pasteurized milk . . . . .	247
MARY F. LEACH: A preliminary study of the sensitizing portion of egg-white . . . . .	253
GEORGE A. OLSON: Milk proteins . . . . .	261
C. A. HERTER and A. I. KENDALL: The use of the fermentation tube in intestinal bacteriology . . . . .	283
C. A. HERTER and A. I. KENDALL: An observation on the fate of <i>B. Bulgaricus</i> (in Bacillac) in the digestive tract of a monkey (Plates I-III) . . . . .	293
H. D. DAKIN: Further studies of the mode of oxidation of phenyl derivatives of the fatty acids in the animal organism. III. Synthesis of some derivatives of phenylpropionic acid . . . . .	303
ALONZO ENGLEBERT TAYLOR: Chemical studies in cytolysis . . . . .	311
ALONZO ENGLEBERT TAYLOR: On the conversion of glycogen into sugar in the liver . . . . .	315
ALONZO ENGLEBERT TAYLOR: On the antagonism of alcohol to carbolic acid . . . . .	319
C. L. ALSBERG and E. D. CLARK: The blood clot of <i>Limulus Polyphemus</i> . . . . .	323
CHARLES HUGH NEILSON and M. H. SCHEELE: The effect of diet on the maltose-splitting power of the saliva . . . . .	331
JULIA T. EMERSON and WILLIAM H. WELKER: Some notes on the chemical composition and toxicity of <i>Ibervillea Sonora</i> (Plates IV and V) . . . . .	339
THEO. C. BURNETT: The inhibiting effect of potassium chloride in sodium chloride glycosuria . . . . .	351
PAUL J. HANZLIK and P. B. HAWK: The uric acid excretion of normal men . . . . .	355

AMOS W. PETERS: Studies on Enzymes; I. The adsorption of diastase and catalase by colloidal protein and by normal lead phosphate . . . . .	367
ALONZO ENGLEBERT TAYLOR: On the synthesis of protamin through ferment action . . . . .	381
ALONZO ENGLEBERT TAYLOR: On the composition and derivation of protamin . . . . .	389
ALONZO ENGLEBERT TAYLOR: On the question of the identity of pepsin and chymosin . . . . .	399
ALONZO ENGLEBERT TAYLOR: On the inversion of cane sugar and maltose by ferments . . . . .	405
H. D. DAKIN: Note on the oxidation of glutamic and aspartic acids by means of hydrogen peroxide . . . . .	409
H. D. DAKIN: The action of glycolcoll as a detoxicating agent . . . . .	413
ARTHUR I. KENDALL: <i>Bacillus Infantilis</i> (n. s.) and its relation to infantilism . . . . .	419
C. A. HERTER and A. I. KENDALL: Note on the products of <i>B. infantilis</i> grown in artificial media (Plates VI and VII) . . . . .	439
J. J. R. MACLEOD: A comparison of the methods of Reid and Schenck for the quantitative estimation of the reducing substances in the blood . . . . .	443
R. H. NICHOLL: The relationship between the ionic potentials of salts and their power of inhibiting lipolysis . . . . .	453
WILLIAM F. BOOS, M.D., Ph.D.: On the reducing component of yeast nucleic acid . . . . .	469
PAUL E. HOWE and P. B. HAWK: Comparative tests of Spiro's and Folin's methods for the determination of ammonia and urea . . . . .	477
STANLEY R. BENEDICT: A reagent for the detection of reducing sugars . . . . .	485
CHRISTIAN A. HERTER: Note on the occurrence of skatol and indol in the wood of <i>Celtis Reticulosa</i> (Miquel) . . . . .	489
T. BRAILSFORD ROBERTSON: On the synthesis of paranuclein through the agency of pepsin and the chemical mechanics of the hydrolysis and synthesis of proteins through the agency of enzymes . . . . .	493
INDEX . . . . .	525



# ON THE IDENTITY OF THE NUCLEIC ACIDS OF THE THYMUS, SPLEEN AND PANCREAS.

By WALTER JONES.

(From the Laboratory of Physiological Chemistry, Johns Hopkins University.)

(Received for publication, May 1, 1908.)

A study of the nucleic acids is conceded to be one of the most puzzling tasks that confront the student of physiological chemistry and for the reason that the literature on the subject reveals a mass of contradictions, corrections and inconsistencies which it would seem almost impossible to reduce to any satisfactory scientific order. As is well known these substances yield among their hydrolytic products various derivatives of purin and the older literature records four substances of this type (guanin, adenin, xanthin and hypoxanthin) any combination of which might be expected among the decomposition products of a nucleic acid. From the fact that certain nucleic acids produced one of these purin derivatives considerably in excess of the other three and that no simple stoichiometric relation was found to exist between the various purin derivatives found in this connection, Kossel advanced his hypothesis of the four nucleic acids each giving one purin base. On the other hand Schmiedeborg contended that salmon nucleic acid contains in the one molecule groups of both guanin and adenin. Investigations which followed Kossel's earlier work have shown that in many instances xanthin and hypoxanthin are secondary products and need not be considered in this connection. Thus it was found that a number of nucleic acids yield guanin and adenin but neither xanthin nor hypoxanthin when prepared from fresh glands and hydrolyzed under conditions which do not admit of the alteration of the purin derivatives first formed. This was shown by Kossel and Neumann<sup>1</sup> to be true of thymus nucleic acid, by Osborne and Harris<sup>2</sup> of triticonucleic acid, by Jones

<sup>1</sup> Kossel and Neumann: *Zeitschr. f. physiol. Chem.*, xxii, p. 74.

<sup>2</sup> Osborne and Harris: *Ibid.*, xxxvi, p. 85.

and Whipple<sup>1</sup> of the nucleic acids of the pancreas and suprarenal gland, and by Levene<sup>2</sup> of a large number of nucleic acids of various origin. The confusions in the older literature are in great measure due to the secondary action of the hydrolytic agent but in part also to the presence in the glands of ferments which liberate the amidopurins from the nucleic acid and convert them into the oxypurins, the latter probably being present as such in the nucleic acid preparations which were employed for a study of the hydrolytic products.<sup>3</sup> At a time when difficulties of this nature seemed to be removed, Steudel<sup>4</sup> made a contribution which unnecessarily threw the question of purin bases again into confusion. He submitted the copper salt of thymus nucleic acid to a severe method of hydrolysis and naturally obtained all four purin bases.

It is not necessary to go beyond Steudel's papers themselves to see wherein the difficulty consisted, but as might have been expected Steudel is now quoted as authority for the statement that thymus nucleic acid produces all four purin bases. However, Jones and Austrian<sup>5</sup> found that no xanthin is produced when thymus nucleic acid is decomposed by the action of the ferment nuclease at the body temperature and in a practically neutral fluid. At the same time Steudel<sup>6</sup> ignored his own findings and stated that both salmon nucleic acid and thymus nucleic acid yield exclusively guanin and adenin and that the two nucleic acids produce these two bases in the same relative quantities. It would seem, therefore, that at the present there is no well established work which is at variance with the general statement that all ordinary nucleic acids whether of plant or animal origin yield two and only two purin derivatives, viz: guanin and adenin.<sup>7</sup>

<sup>1</sup> Jones and Whipple: *Amer. Journ. of Physiol.*, vii, p. 423.

<sup>2</sup> Levene: *Zeitschr. f. physiol. Chem.*, xxxvii, p. 402; xxxix, p. 479; xlv, p. 370; l, p. i; Levene and Mandel; *Ibid.*, xlvi, p. 155; xlvii, p. 140; xlix, p. 262.

<sup>3</sup> Jones and Austrian: *Ibid.*, xlviii, p. 110.

<sup>4</sup> Steudel: *Ibid.*, xlii, p. 165.

<sup>5</sup> Jones and Austrian: *This Journal*, iii, p. 1.

<sup>6</sup> Steudel: *Zeitschr. f. physiol. Chem.*, xlix, p. 406; liii, p. 14.

<sup>7</sup> The expression "ordinary nucleic acid" is used in this paper in its narrow sense and does not include the "guanylic acids" which form a distinct class of themselves.

Our ideas in regard to pyrimidin derivatives have undergone similar changes. Thymin was first obtained from thymus nucleic acid by Kossel and Neumann<sup>1</sup> and their selection of this term leads to the suspicion that they regarded the compound as characteristic of the gland. But thymin has been frequently found among the hydrolytic products of nucleic acids<sup>2</sup> and had come to be regarded as a constant decomposition product when Ascoli<sup>3</sup> observed that in yeast nucleic acid the thymin group is replaced by a uracil group. Osborne and Harris<sup>4</sup> showed the same to be true of the nucleic acid of the wheat embryo while Mandel and Levene<sup>5</sup> obtained uracil but no thymin from the nucleic acid of haddock eggs. Following Ascoli's discovery Levene obtained uracil (in addition to thymin) from various animal nucleic acids but finally ascribed the substance to secondary action. This position is now taken by Steudel with reference to uracil in general.

In 1894 Kossel and Neumann<sup>6</sup> obtained from thymus nucleic acid an organic base which they called cytosin and which was subsequently proven to be a pyrimidin derivative.<sup>7</sup> In the meantime Levene<sup>8</sup> showed that spleen nucleic acid gives rise to a base having the empirical composition of amido-oxy-pyrimidin. He afterwards obtained the substance from a number of nucleic acids and was able to prove its identity with cytosin.

Again, Kossel<sup>9</sup> showed that lævulinic acid results from the hydrolysis of thymus nucleic acid and as the substance is not formed from pentose Kossel's discovery indicates a hexose group in this nucleic acid molecule. Subsequent work by Levene<sup>10</sup> failed to establish the presence of this group in other nucleic acids and it seemed for a time that thymus nucleic acid was

<sup>1</sup> Kossel and Neumann: *Ber. d. deutsch. chem. Gesellsch.*, xxvi, p. 2753.

<sup>2</sup> Levene: *Loc. cit.*, and *Zeitschr. f. physiol. Chem.*, xxxix, pp. 4 and 133.

<sup>3</sup> Ascoli: *Zeitschr. f. physiol. Chem.*, xxviii, p. 426.

<sup>4</sup> Osborne and Harris: *Loc. cit.*

<sup>5</sup> Mandel and Levene: *This Journal*, i, p. 425.

<sup>6</sup> Kossel and Neumann: *Ber. d. deutsch. chem. Gesellsch.*, xxvii, p. 2215.

<sup>7</sup> Kossel and Steudel: *Zeitschr. f. physiol. Chem.*, xxxviii, p. 49.

<sup>8</sup> Levene: *Ibid.*, xxxvii, p. 402.

<sup>9</sup> Kossel: *Arch. f. Physiol.*, p. 157, 1893; Noll: *Zeitschr. f. physiol. Chem.*, xxv, p. 430.

<sup>10</sup> Levene: *Zeitschr. f. physiol. Chem.*, xxxvii, p. 402.



thus shown to have a point of distinction from other members of this group, but by the adoption of new methods Levene<sup>1</sup> convinced himself of the presence of the lævulinic acid group in several nucleic acids which he examined.

When we consider the quantitative differences presented by the decomposition products of nucleic acids we notice the same two opposite tendencies, i. e., a number of researches indicate that all nucleic acids yield the same relative quantities of their decomposition products, and on the other hand an equal number seem to prove that this relation is different for the various nucleic acids. Moreover, this phase of the subject has become complicated to a most unusual degree by the presence of guanylic acid in the tissues. Ivar Bang<sup>2</sup> discovered this substance in the pancreas. He stated that it yields glycerin phosphoric acid, furfurol and guanin but neither adenin nor any pyrimidin derivative. The matter of glycerin phosphoric acid seems to be discredited<sup>3</sup> but the other points in connection with the substance have been sufficiently confirmed. When it is remembered that substances of the guanylic acid type are not confined to the pancreas<sup>4</sup> it will appear that the presence of this substance in varying quantities might account for some at least of the differences presented by preparations supposed to be exclusively "ordinary nucleic acids."

Thus while the weight of recent evidence has inclined to the belief that certain supposedly different nucleic acids are the same substance and while the support for these supposed differences has been much weakened or entirely removed, a considerable amount of positive evidence has accumulated to show the chemical identity of certain nucleic acids from sources zoologically distant from one another. Herlant<sup>5</sup> has prepared specimens of nucleic acid from the thymus and from fish spermatozoa and has shown that the two preparations have the same chemical composition.

<sup>1</sup> Levene: *Zeitschr. f. physiol. Chem.*, xliii, p. 109.

<sup>2</sup> Bang: *Ibid.*, xxvi, p. 133; xxxi, p. 411.

<sup>3</sup> Steudel: *Ibid.*, liii, p. 539.

<sup>4</sup> Jones and Rowntree: *This Journal*, iv, p. 289.

<sup>5</sup> Herlant: *Arch. f. exp. Pathol. u. Pharmacol.*, xlv, p. 148.

Again Steudel<sup>1</sup> has lately proposed a very accurate method of estimating the purin derivatives obtainable from a nucleic acid. The substance is treated with concentrated nitric acid when a violent reaction ensues with evolution of the brown oxides of nitrogen and on cooling the products, guanin and adenin are quantitatively deposited as crystalline nitrates. The removal of the two nitrates from the other decomposition products and their separation from one another can be accomplished with great accuracy, and in this manner Steudel has obtained identical results from the nucleic acids of the thymus gland and salmon testicle.<sup>2</sup>

Finally, Schmiedeberg<sup>3</sup> in a critical review concludes that a large number of nucleic acids of various origin which have been examined in his laboratory are identical chemical substances. It is, therefore, probable that the following summary of the nucleic acid question is not far from correct.

(1) All "ordinary nucleic acids" of plant or animal origin yield the same two purin derivatives (guanin and adenin) and in the same quantitative ratio.

(2) All "ordinary nucleic acids" yield cytosin.

(3) All "ordinary nucleic acids" yield lævulinic acid.

<sup>1</sup> Steudel: *Zeitschr. f. physiol. Chem.*, xlviii, p. 425.

<sup>2</sup> This work of Steudel appears to me a little curious. I have had frequent occasion to use the method both before and after Steudel's announcement and can agree with him in regard to the quantitative ratio of the guanin and adenin groups in thymus nucleic acid. But one will obtain no trace of either guanin or adenin in the crystalline deposit when the method is executed as described. The product is a mixture of xanthin and hypoxanthin nitrate and I base my conclusion regarding the guanin and adenin groups of the nucleic acid on the relative quantities of xanthin and hypoxanthin obtained. One would scarcely expect to find either guanin or adenin after a violent evolution of the oxides of nitrogen and as a matter of fact no trace of either is to be found. Nucleic acid may be hydrolyzed with diluted nitric acid from which all traces of the oxides of nitrogen have been removed and nitrates of guanin and adenin be obtained. But under these conditions there is no evolution of the oxides of nitrogen and the deposition of the nitrates is very slow, requiring several days for completion. Steudel specifically states *concentrated nitric acid* describes the violence of the reaction, and repeats *concentrated nitric acid* in subsequent references. (*Zeitschr. f. physiol. Chem.*, liii, p. 14.)

<sup>3</sup> Schmiedelberg: *Arch. f. exp. Pathol. u. Pharmakol.*, lvii, p. 309.

(4) Nucleic acids from plants and fish eggs yield uracil while those from animal organs and fish spermatozoa yield thymine.

(5) There is considerable evidence for regarding the nucleic acids of different mammalian organs as identical substances, and there is no insurmountable evidence against the assumption. The statements in the literature which are at variance with these conclusions are satisfactorily explained on the following grounds.

(6) In some cases where it was difficult to obtain material the experimenter has endeavored to study the purin and pyrimidin derivatives with the same specimen, and in order to insure the complete hydrolysis of the nucleic acid, hydrolytic agents have been employed which remove amido groups from both purin and pyrimidin rings.<sup>1</sup> In this manner, xanthin hypoxanthin and uracil have resulted by secondary action from guanine, adenine and cytosine.

(7) The presence in the tissues of "amidases" was not known to the earlier investigators, who in many cases must have worked with glands that had stood for a time and in which the removal of amido groups had proceeded to a considerable extent.

(8) There exists in various glands a curious nucleic acid (if it be properly so called) which yields an excessive amount of both pentose and guanine. This substance may well be responsible for the furfural reaction given by ordinary nucleic acids and has undoubtedly produced a confusion in regard to the excessive amount of guanine supposed to result from the hydrolysis of some members of the class of ordinary nucleic acids.<sup>2</sup>

The object of this communication is to describe some experiments with preparations of ordinary nucleic acid of various origin (spleen and pancreas of the pig and thymus of the calf) which have been obtained in such a manner as to avoid all errors from any source that has been mentioned above, and to furnish what should be considered conclusive evidence of the identity of these particular nucleic acids. Upon finding that guanilic acid is not confined to the pancreas but is present in the spleen, the mammary gland and probably in other organs, various

<sup>1</sup> Inouye: *Zeitschr. f. physiol. Chem.*, xlviii, p. 181; Inouye Katsuji and Katoke: *Ibid.*, xlii, p. 201.

<sup>2</sup> Jones and Whipple: *Loc. cit.*

glands were first treated in such a manner as to remove the guanylic acid and ordinary nucleic acids were then prepared from the residual material. While the primary object of this procedure was the removal of guanylic acid (i. e., its nucleoproteid) it has the great advantage of removing other objectionable constituents of the glands and especially coloring matter so that the ordinary nucleic acids obtained from the residues yield sodium salts which are peculiarly adapted to polarimetric measurement. Thus the nucleic acid of the spleen which when prepared directly from the organ is always somewhat colored, when prepared by this new procedure is so nearly colorless that the polarimetric measurement of a 4 per cent solution in a 2 decimeter tube offers no greater difficulty than an empty tube.

Various nucleic acids prepared in this manner have the following points in common:

(1) Their neutral sodium salts are all dextrorotatory and all have the same specific rotation.

(2) The variation of the rotation with concentration is in each case according to the same law.

(3) The variation of the rotation by the addition of ammonia or alkalis is according to the same law in the case of each nucleic acid but different from the variation by dilution with water.

(4) The variation of the rotation of the neutral sodium salt by the addition of acids obeys a third law which, however, is the same for each nucleic acid.

(5) All three of the nucleic acids examined, form gelatinous neutral sodium salts.

(6) In the case of all three preparations the gelatinous character of the neutral sodium salt disappears upon the addition of either acid or alkali and the specific rotation changes accordingly. This easy passage of the gelatinous into the non-gelatinous form and the reverse offers a simple explanation of the physiological localization and migration of nucleic acid.

(7) It is intended to confine this paper to the polarization and gelatinization of the sodium salts of the three nucleic acids mentioned, yet it may be stated here that all three preparations yield both guanin and adenin and in relative quantities which were found to agree as closely as could be expected from the methods at our disposal.

PREPARATION OF ORDINARY NUCLEIC ACIDS FROM SPLEEN, THYMUS  
AND PANCREAS.

The best specimens of the nucleic acids of spleen and pancreas have been prepared and analyzed by Levene. In his method a solution of the nucleic acid in dilute acetic acid was treated with picric acid for the removal of the proteids and the nucleic acid was precipitated from the filtered solution by the addition of copper chloride. The method is an excellent one for the elimination of carbohydrates and other constituents of glands which were known at the time the method was proposed but whether by this process guanylic acid is removed is still a question. Levene found spleen nucleic acid characterized by the relatively large amount of cytosin which it yields. Thymus nucleic acid has been the subject of a large number of researches but no one has prepared a nucleic acid from this gland by the method to be described. I make no claim that this gland contains guanylic acid but in case it does the substance will surely be removed by the method described. Steudel has recently confirmed the existence of guanylic acid in the pancreas and states that after the removal of this substance the residue answers the requirements of Neumann's method and yields an ordinary nucleic acid which produces both guanin and adenin but which differs from thymus nucleic acid in that its sodium salt does not gelatinize. I have not observed such a difference. The neutral sodium salt of pancreas nucleic acid forms a 6 per cent solution which on cooling to the room temperature gelatinizes so solidly that the vessel may be inverted without losing its contents. Nor do I understand that Neumann was accustomed to boil out his glands with water before extracting the nucleic acid. He boils with a very dilute acetic acid solution which is done for the coagulation of the proteids so that the glands can afterwards be finely ground. Such a procedure with the pancreas or spleen would of course shut in the guanylic acid which latter would surely contaminate the final nucleic acid preparation.

The method of preparation which was employed for the material described below was essentially the same for all three glands and may be briefly described as follows: Portions of ground gland of one kilo each were well mixed with distilled

water at the room temperature and the mixture brought slowly to the boiling point. The fluid was roughly decanted from the coagulum for the removal of all suspended as well as soluble material and the latter was freed as far as possible from the fluid by pressing with linen. The residue was again finely ground in a machine for the purpose, boiled out with  $2\frac{1}{2}$  parts of distilled water, and the aqueous fluid roughly decanted and pressed out with linen. The grinding and extraction with water was repeated a third time. The final residue was added in small successive portions to a solution of 100 grams of sodium acetate and 30 grams of caustic soda in 2 liters of water which had been previously heated to the boiling point and which was kept hot during the addition of the gland.

After the gland residue had all been added to the alkaline solution the product was kept just below the boiling point for 20 minutes and while still hot was treated with 25 per cent acetic acid until the fluid failed to give any indication with red litmus. This is considerably past the point where the fluid begins to turn blue litmus. A fluid brought to this condition of acidity will usually filter rapidly, giving a perfectly clear filtrate and it is necessary that this condition be reached either by testing with litmus or by frequent attempts at filtration with small quantities of fluid and subsequent addition of small amounts of either acid or alkali as the case requires. The filtration is best accomplished with a hot water funnel, since the phosphates are precipitated as the solution cools and soon stop an ordinary filter. With the thymus gland the hot water funnel is necessary to prevent gelatinization, since we are here dealing with a more concentrated solution of nucleic acid. In any case the filtrate is exactly neutralized and evaporated to about 700 cc. (in case of the pancreas to a smaller volume) and while warm poured into an equal volume of alcohol. A snow-white, voluminous precipitate of the neutral sodium salt of nucleic acid is thrown out which on standing adheres to the bottom and sides of the vessel so that the colored alcoholic fluid may be completely decanted. The product was washed in turn with alcohol of increasing concentration until 95 per cent alcohol was employed, when the material was pressed as dry as possible with linen and dissolved in 200 cc. of hot 1 per cent caustic soda. At this point differ-

ent procedures were adopted with different preparations, the heating of the alkaline solution being discontinued in some cases as soon as the phosphates had separated, leaving a clear interstitial fluid while in other cases the heating of the alkaline solution on the water bath was continued for six hours. This variation was adopted after some knowledge of the optical properties of the products had been acquired and its object was to see what influence the heating with dilute alkalis would have on these optical properties and also on the gelatinization of the neutral sodium salts. The results will show that the alkaline treatment has no influence in either respect.

The alkaline fluid was filtered from the phosphates (there is no necessity to use a hot water funnel at this point) and exactly neutralized with acetic acid. When the neutral point is approached the fluid becomes viscous and just before it is reached the material stiffens to a solid jelly even in the warm, and this is the case no matter what the length of time during which the substance was heated with alkali. The neutral salt gelatinizes, the other salts do not. It is, therefore, necessary to make the final neutralization with a hot solution. The neutral fluid is poured into alcohol and the sodium salt dried with alcohol and ether as before described. This product dissolves in water to a practically colorless solution but an insignificant amount of insoluble phosphate gives the faintest indication of a cloud. The material was, therefore, made into a 6 per cent solution in water and allowed to stand over night. The cloud collected to a well defined flocculent deposit which was easily filtered off (after warming the solution) leaving a perfectly transparent, almost colorless fluid. This was poured into alcohol and the product ground in a mortar with successive portions of alcohol, which were used for drying. In this way the material is brought to a fine state of division so that the final product after the use of ether falls immediately to a perfectly dry dust-like powder which has no odor of alcohol. In order to be sure of equally dry preparations, this material was not allowed to stand before use, but portions were immediately weighed out and made up into solutions intended for the polarimetric work that follows.

A description of any one of the three preparations will answer

equally well for the other two. All are snow-white, dustlike, non-hygroscopic powders, which form aqueous solutions that are perfectly transparent, practically colorless, and neutral to litmus. Even concentrated aqueous solution gave only an opalescence with alcohol in equal volume, but the addition of sodium acetate in traces causes the sudden appearance of a flocculent precipitate leaving a clear interstitial liquid. None of the preparations give any response to the biuret reaction even when made by the accurate method which Schmiedeberg has lately proposed.<sup>1</sup> A 2 per cent solution has little viscosity at the room temperature; a  $2\frac{1}{2}$  per cent solution is about as mobile as concentrated sulphuric acid; a 3 per cent solution drops very slowly from a burette with stopcock open and gelatinizes on standing over night in a cool room, and a .5 per cent solution gelatinizes completely as soon as the room temperature is reached.

#### THE OPTICAL PROPERTIES OF THE ORDINARY NUCLEIC ACIDS OF THE THYMUS, PANCREAS AND SPLEEN.

Preliminary experiments had shown that on account of the gelatinous property of the sodium salts, the maximum concentration which could be satisfactorily employed was a 4 per cent solution. Solutions of this concentration were prepared and the more dilute solutions were obtained from this by the addition of water (or reagent) in the quantities indicated in the tables. This dilution cost some trouble owing to gelatinization. The original 4 per cent solution may be brought to the room temperature and allowed to stand for an hour or so without solidifying but on standing several hours gelatinization occurs and the material must be warmed for further use. In making up the 4 per cent solution, the material was usually dissolved in hot water and transferred to a measuring flask, which was filled nearly to the neck with hot water and the materials mixed as well as possible while still hot. The solution was allowed to stand over night and filled to the mark with cold water. The gelatinous material in the bottom of the flask was then melted by slight warming and the fluids thoroughly mixed. The dilution was afterwards done with both water and nucleic acid solu-

<sup>1</sup> Schmiedeberg: *Loc. cit.*



tion at a temperature slightly above that of the room. The polarizations were made in 2 dm. tubes and the angles recorded are in each case the mean of five readings. In the cases of the more dilute solutions the recorded angle is the mean of a large number of readings selected near the mean,

The observations recorded in the tables were made with the three neutral sodium salts of the nucleic acids of the thymus, spleen and pancreas. For studying the effects of heating thymus nucleic acid with dilute alkalis a series of experiments were made with a specimen of sodium salt of this nucleic acid which had been heated on the water bath for 6 hours with 1 per cent caustic soda. This specimen is designated in the tables as "Thymus II."

As will be seen the rotation of each sodium salt varies with the concentration and also by the addition of acids or alkalis. The laws of these variations are brought out more clearly in the diagrams which have been constructed from the tables. In these diagrams ordinates represent the observed angles of rotation divided by half the percentages of the solutions. This value which has been used for its convenient magnitude is of course a function of the specific rotation  $[\frac{\alpha_D}{c}]$  and if the rotation of the substances did not vary under the conditions employed, all curves would be straight horizontal lines. Abcissas represent the percentages of the solutions multiplied by five-thirds.

Diagram I shows curves of dilution with water. All three curves are slightly "convex upward," although they are nearly straight lines. The irregularities near the origin are to be expected where one is dealing with such dilute solutions that an error of 2' in reading the polarimeter would make a considerable difference in the location of the point on the diagram. The spleen and thymus curves are practically coincident while the slight but uniform departure of the pancreas curve might reasonably be attributable to an error in making up the original 4 per cent solution. This assumption is not borne out, however, by the pancreas curves in the diagrams which follow. The pancreas solutions were all allowed to stand over night before polarization while the spleen and thymus solutions were not. A slight multirotation or change in temperature might therefore account for the small difference. The points of interest are that the

substances that are identical even to their space relations. It is remotely possible that the small differences observed are to be attributed to slight differences in chemical constitution between the nucleic acids but it is more reasonable to assume that they represent deficiencies in method.

#### ON GELATINOUS AND NON-GELATINOUS SALTS OF NUCLEIC ACIDS.

In 1898 Neumann<sup>1</sup> found it possible to prepare from the thymus gland two different nucleic acids. The one of these ( $\alpha$ -nucleic acid) forms a sodium salt whose solution in warm water gelatinizes on cooling while the sodium salt of the other ( $\beta$ -nucleic acid) exhibits no such property. Neumann did not believe that these two acids existed independently of one another in the gland but that the  $\beta$ -acid is formed at the expense of the  $\alpha$ -acid by the action of the hot caustic soda which he used in his method of preparation. The existence of these two thymus nucleic acids has been accepted by later investigators and it has usually been granted that thymus nucleic acid is characterized as the sole nucleic acid which yields a gelatinous sodium salt. Thus Steudel<sup>2</sup> describes a nucleic acid of the pancreas as very similar to thymus nucleic acid but different from the latter in that its sodium salt does not gelatinize. Kostytschew<sup>3</sup> by a study of the barium salts confirmed Neumann's discovery of the two thymus nucleic acids, and Araki<sup>4</sup> professed to demonstrate the existence in the thymus of a ferment which causes the conversion of the  $\alpha$ -nucleic acid into the  $\beta$ -nucleic acid.

Schmiedeberg remarks in his recent communication<sup>5</sup> that he has frequently noticed the tendency to gelatinize on the part of copper compounds of various nucleic acids, a property which was lost when the copper compounds were further treated with chemical reagents. While he does not claim to have discovered the relation between the  $\alpha$  and  $\beta$  acids he denies that there is any essential difference between the two and expresses the opinion that the one acid is simply the anhydride of the other.

<sup>1</sup> Neumann: *Arch. f. Physiol*, suppl., p. 552, 1899.

<sup>2</sup> Steudel: *Zeitschr. f. physiol. Chem.*, liii, p. 539.

<sup>3</sup> Kostytschew: *Ibid.*, xxxix, p. 544.

<sup>4</sup> Araki: *Ibid.*, xxxviii, p. 98.

<sup>5</sup> *Loc. cit.*

Schmiedeberg's denial of the independent existence of the two thymus nucleic acids is perfectly correct, but the explanation of the relation between the gelatinous and non-gelatinous sodium salts is even simpler than the one which he proposes. We are in fact here dealing merely with two different sodium salts of the same acid.

It has been shown above that the optical properties of the sodium salt of thymus nucleic acid prepared by moderate treatment with alkali do not differ from those of a sodium salt prepared by excessive treatment with alkali, and yet this one difference in the method of preparation has been supposed to be the determining factor in the production of the two substances. It may now be added that there is no easily observed difference in the viscosity of solutions of the two preparations with which the polarimetric work was done. This was determined by dropping the two solutions in succession from the same burette with open stopcock and counting the drops that fall in a given time. The differences noted in a number of experiments were no greater than were noted in the polarimetric work.

Thymus nucleic acid is evidently a polybasic acid which forms several series of salts. One of those salts has been called in this paper the neutral salt on account of the behavior of its aqueous solution to litmus. This salt (which properly should be called an acid salt) is the one which gelatinizes easily in a 5 per cent solution and shows the maximum rotation to polarized light. If a trace of either acid or alkali be added to a solution of this salt evidently the relation between base and nucleic acid is disturbed, a more acid, a less acid or a neutral salt is formed, as the case may be, the specific rotation falls and the *gelatinous character is lost*. Abundant opportunity to notice the loss of gelatinization in the dilution of solutions with acid and ammonia was offered by the materials used in the polarimetric work.

In precipitating the sodium salt from its aqueous solution with alcohol, the non-gelatinous form will be obtained unless the aqueous solution is neutral to litmus. This non-gelatinous salt will not react neutral to litmus and will show a low specific rotation. On dissolving this salt in water neutralizing the solution and again precipitating with alcohol the gelatinous salt is obtained. It happened on several occasions that a preparation

which was intended for polarimetric measurement did not possess the proper gelatinization and its rotation was found too low. On treatment with ammonia the rotation *rose in the beginning* instead of falling. A little alkali was added to the solution when the viscosity was increased so that the solution gelatinized solidly on standing and the rotation rose to the proper point. This phenomenon has been occasionally observed with preparations that were neutral to litmus so that it must be concluded that in dealing with these salts the rotation and gelatinization are more sensitive means of determination than is litmus. These points relative to gelatinization apply equally well to all three nucleic acids here under discussion.

Finally, a number of preparations of thymus nucleic acid which were made years ago were examined. These materials were labeled "gelatinous sodium salt" and "non-gelatinous salt," in accordance with the method that had been employed in their preparation. But many of those called non-gelatinous were found gelatinous and vice versa. Those which actually gelatinized showed a higher rotation than the others and the one salt could be converted into the other by the method already suggested.

The various chemical differences between the animal nucleic acids of different origin may of course be due to differences in the chemical constitution of the nucleic acids themselves, but the history of the development of the subject suggests that some at least and perhaps all of those differences are due to the great difficulty of making chemical examinations especially in regard to the relative amounts of the decomposition products. It is difficult to understand how the three nucleic acids described in this paper could possess the same specific rotation which varies in each case according to the same law in whatever way the variation is produced, or how the sodium salts could show the same degree of viscosity which disappears and reappears under precisely the same conditions unless the three nucleic acids in question are identical.

TABLE I.  
Showing variation in rotation with concentration.

	DILUTION.		Percentage.	THYMUS I (').		THYMUS II (').		SPLEEN (°).		PANCREAS (X).	
	Solution.	Water.		Observed angle $\alpha$ .	$\frac{2\alpha}{\text{per cent.}}$	Observed angle $\alpha$ .	$\frac{2\alpha}{\text{per cent.}}$	Observed angle $\alpha$ .	$\frac{2\alpha}{\text{per cent.}}$	Observed angle $\alpha$ .	$\frac{2\alpha}{\text{per cent.}}$
1	cc. original	cc. solution	4.00	5.90°	2.95	5.95°	2.98	5.80°	2.90	6.08°	3.04
2	13.6	2.4	3.40	4.90	2.88	4.85	2.85	4.76	2.80	4.96	2.92
3	10.5	4.5	2.80	3.78	2.70	3.79	2.71	3.74	2.67	3.92	2.80
4	8.8	7.2	2.20	2.75	2.50	2.75	2.50	2.72	2.47	2.90	2.64
5	6.0	9.0	1.60	1.84	2.30	1.84	2.30	1.83	2.29	1.88	2.35
6	4.0	12.0	1.00	1.02	2.04	1.01	2.02	1.00	2.00	1.06	2.12
7	3.0	17.0	0.60	0.57	1.90	0.57	1.90	0.55	1.80	0.60	2.00
8	2.0	18.0	0.40	0.33	1.65	0.3	1.65	0.36	1.80		

TABLE II.  
*Showing variation in rotation by dilution with 5 per cent acetic acid.*

	DILUTION.		Percentage.	THYMUS I (').		THYMUS II (').		SPLEEN (°).		PANCREAS (X).	
	Solution.	Acetic acid.		Observed angle $\alpha$ .	$\frac{2\alpha}{\text{per cent.}}$	Observed angle $\alpha$ .	$\frac{2\alpha}{\text{per cent.}}$	Observed angle $\alpha$ .	$\frac{2\alpha}{\text{per cent.}}$	Observed angle $\alpha$ .	$\frac{2\alpha}{\text{per cent.}}$
1	cc. original	cc. solution	4.00	5.90°	2.95	5.95°	2.98	5.80°	2.90	6.08	3.04
2	13.6	2.4	3.40	4.25	2.50	4.33	2.55	4.18	2.46	4.25	2.50
3	10.5	4.5	2.80	2.87	2.05	2.95	2.11	2.90	2.07	2.81	2.01
4	8.8	7.2	2.20	1.89	1.72	1.87	1.70	1.91	1.74	1.79	1.63
5	6.0	9.0	1.60	1.18	1.48	1.17	1.45	1.16	1.45	1.13	1.41
6	4.0	12.0	1.00	0.61	1.25	0.60	1.20	0.57	1.14	0.55	1.10
7	3.0	17.0	0.60	0.31	1.03	0.33	1.10	0.29	0.97	0.28	0.93
8	2.0	18.0	0.40	0.19	0.95	0.16	0.80	0.17	0.85	0.18	0.90

TABLE III.  
Showing variation in rotation by dilution with 2½ per cent ammonia.

	DILUTION.		Percentage.	THYMUS I (').		THYMUS II (').		SPLEEN (°).		PANCREAS (X).	
	Solution.	Ammonia.		Observed angle α.	$\frac{2\alpha}{\text{per cent.}}$	Observed angle α.	$\frac{2\alpha}{\text{per cent.}}$	Observed angle α.	$\frac{2\alpha}{\text{per cent.}}$	Observed angle α.	$\frac{2\alpha}{\text{per cent.}}$
1	cc. original solution	cc.	4.00	5.90°	2.95	5.95°	2.98	5.80°	2.90	6.08°	3.04
1a	15.2	0.8	3.80	4.77	2.51	4.83	2.54	4.60	2.42	4.66	2.50
1b	14.4	1.6	3.60	3.98	2.21	4.01	2.23	3.94	2.19	3.82	2.12
2	13.6	2.4	3.40	3.40	2.00	3.35	1.97	3.09	1.82	3.06	1.80
3	10.5	4.2	2.80	2.03	1.45	2.13	1.52	1.92	1.37	1.89	1.35
4	8.8	7.2	2.20	1.10	1.00	1.21	1.10	1.06	0.96	1.09	0.99
5	6.0	9.0	1.60	0.68	0.85	0.73	0.91	0.62	0.78	0.65	0.81
6	4.0	12.0	1.00	0.40	0.80	0.45	0.90	0.36	0.72	0.36	0.72
7	3.0	17.0	0.60	0.26	0.87	0.25	0.83	0.21	0.70	0.23	0.77
8	2.0	18.0	0.40	0.18	0.90	0.18	0.90	0.13	0.65	0.16	0.80

TABLE IV.  
*Showing variation in rotation by dilution with 5 per cent ammonia.*

	DILUTION.		Percentage.	THYMUS I (').		THYMUS II (').		SPLEEN (°).		PANCREAS (X).	
	Solution.	Ammonia.		Observed angle $\alpha$ .	$\frac{2\alpha}{\text{per cent.}}$	Observed angle $\alpha$ .	$\frac{2\alpha}{\text{per cent.}}$	Observed angle $\alpha$ .	$\frac{2\alpha}{\text{per cent.}}$	Observed angle $\alpha$ .	$\frac{2\alpha}{\text{per cent.}}$
1	cc. original	cc. solution	4.00	5.90°	2.95	5.95°	2.98	5.80°	2.90		
1a	15.2	0.8	3.80	3.63	1.91	3.85	2.03	3.61	1.90		
1b	14.4	1.6	3.60	2.97	1.65	2.92	1.62	2.91	1.62		
2	13.6	2.4	3.40	2.50	1.47	2.55	1.50	2.43	1.43		
3	10.5	4.5	2.80	1.62	1.16	1.58	1.13	1.58	1.13		
4	8.8	7.2	2.20	0.97	0.88	1.05	0.95	0.96	0.88		
5	6.0	9.0	1.60	0.56	0.70	0.62	0.78	0.62	0.78		
6	4.0	12.0	1.00	0.32	0.64	0.37	0.74	0.37	0.74		
7	3.0	17.0	0.60	0.20	0.67	0.20	0.67	0.23	0.77		
8	2.0	18.0	0.40	0.12	0.60	0.14	0.70	0.16	0.80		



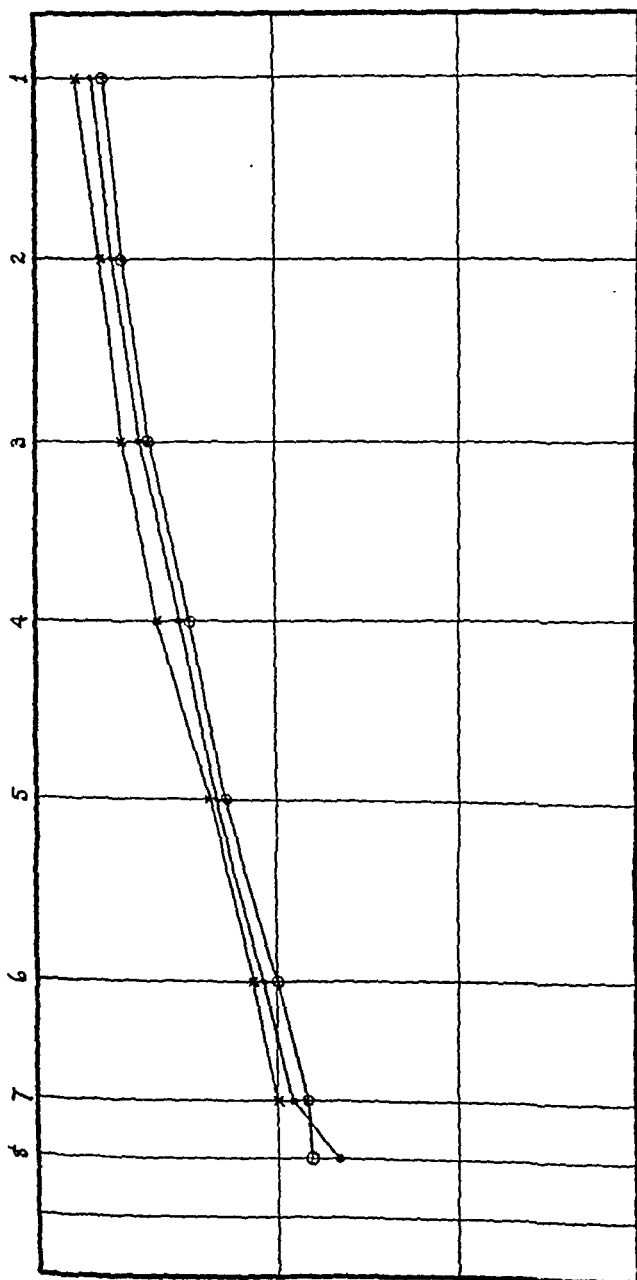


DIAGRAM I.

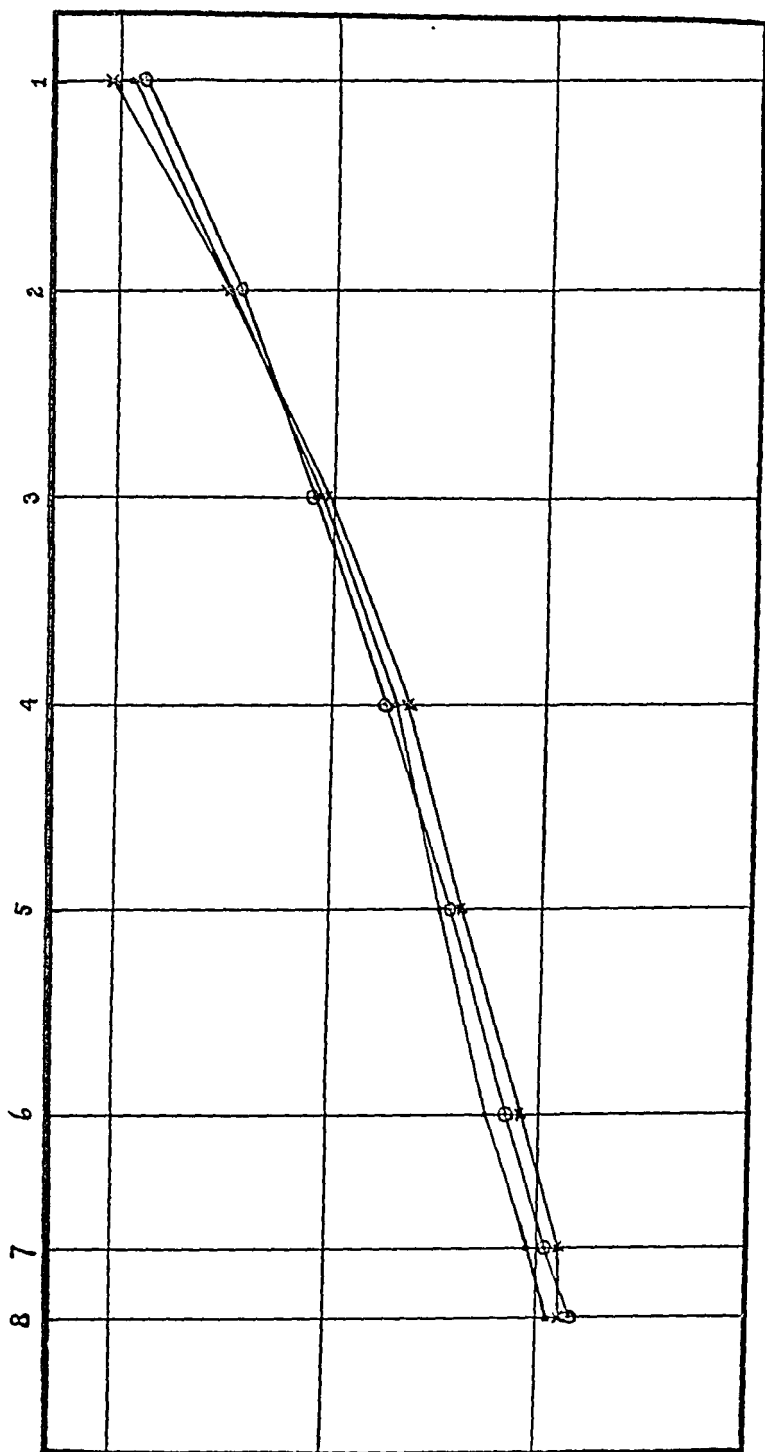


DIAGRAM II.

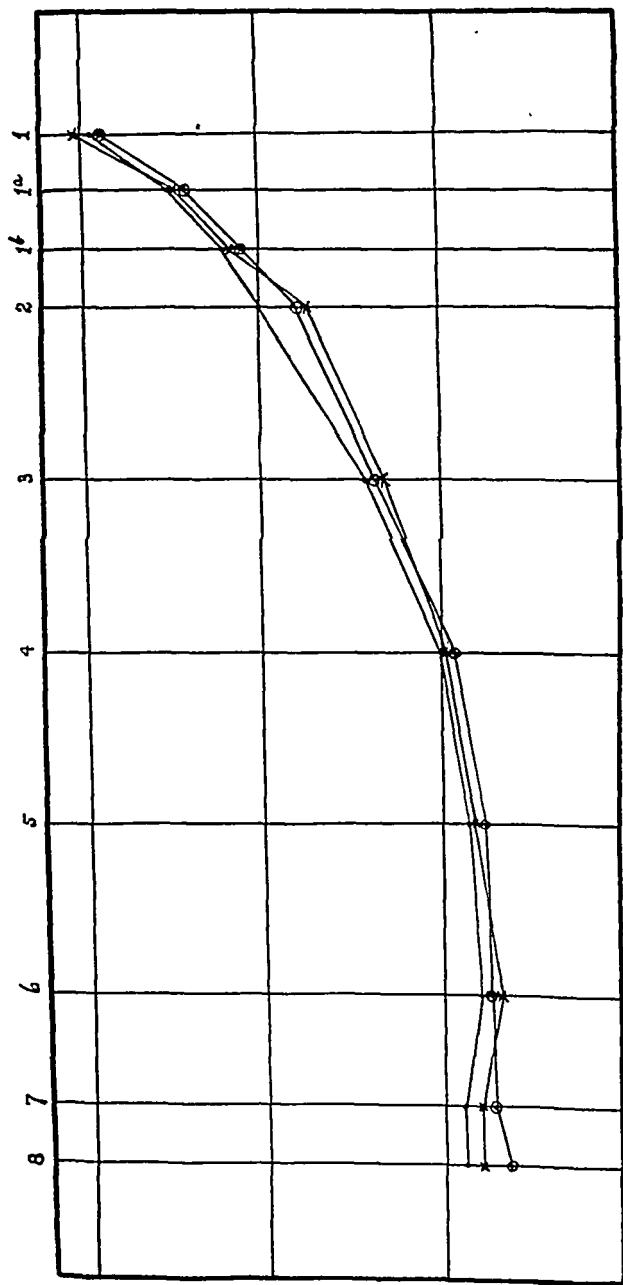


DIAGRAM III.

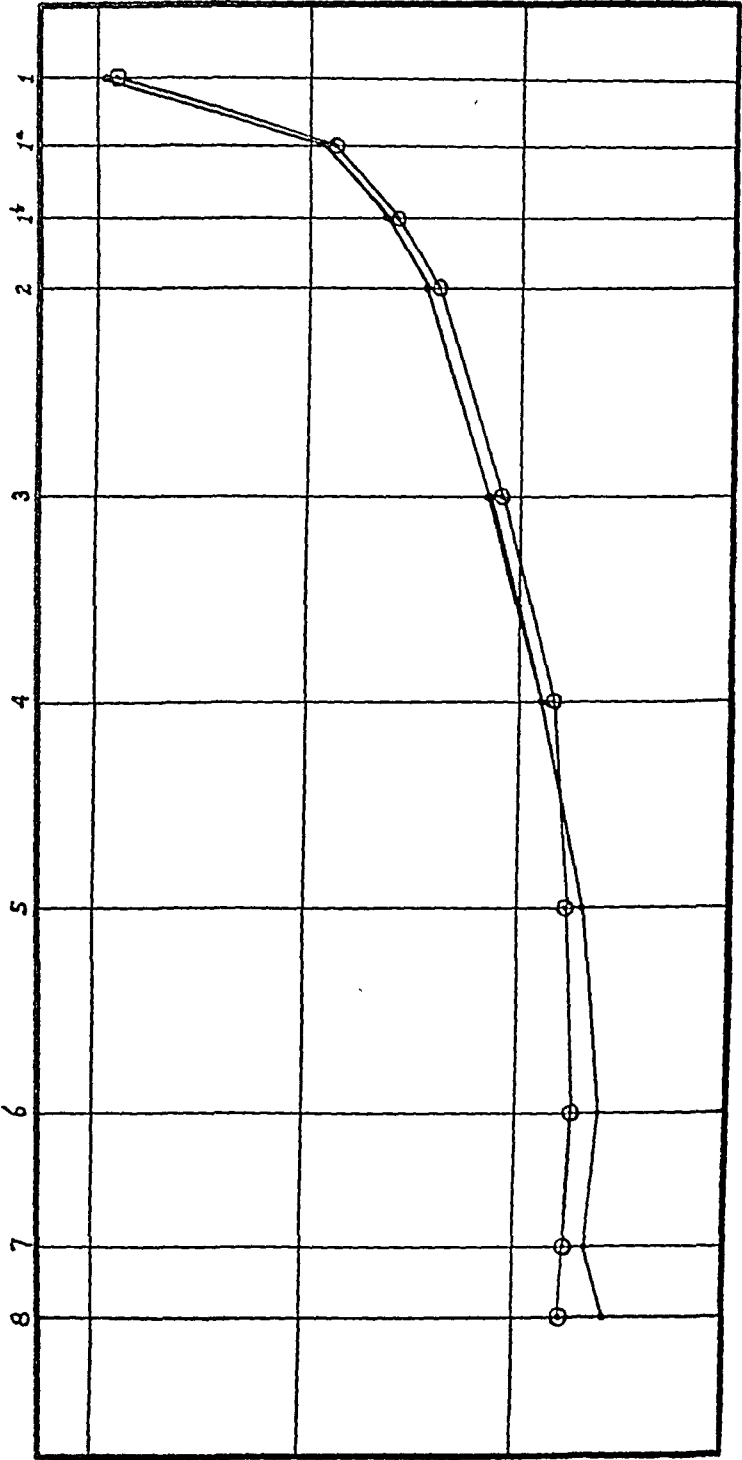


DIAGRAM IV.

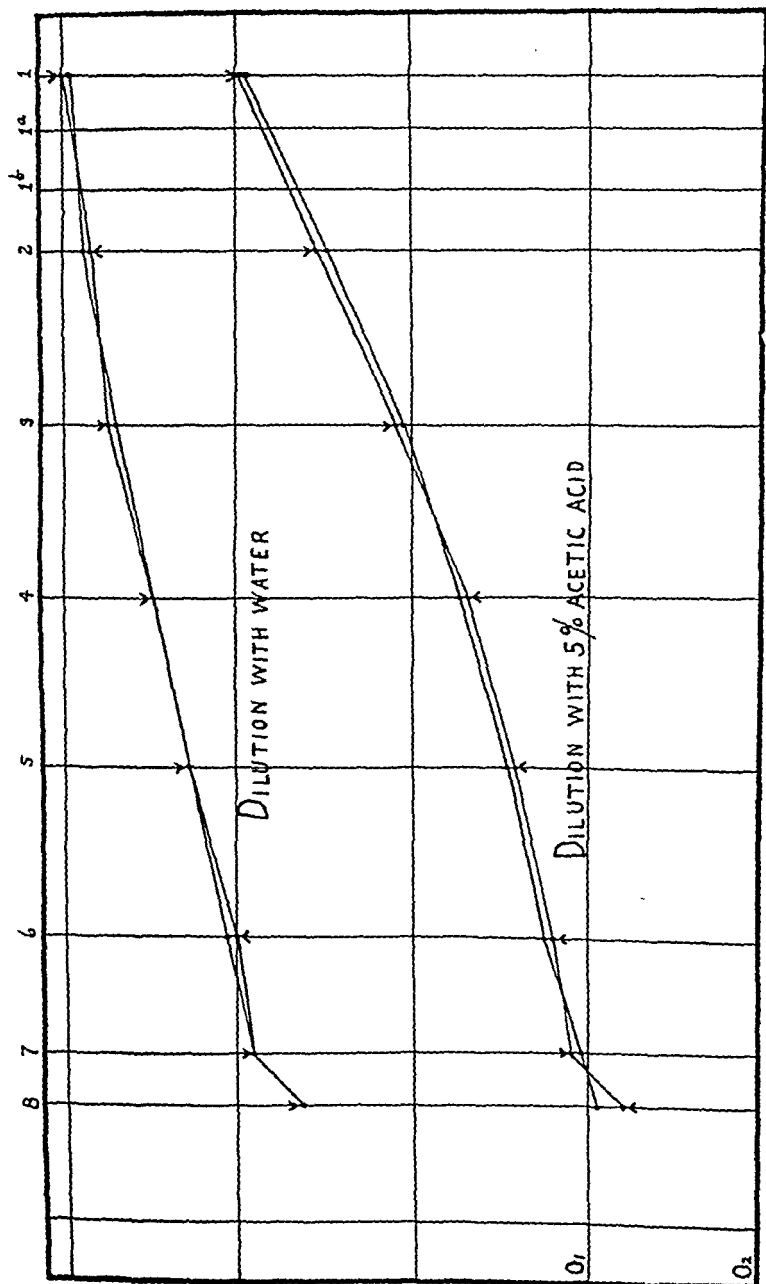


DIAGRAM V-A.

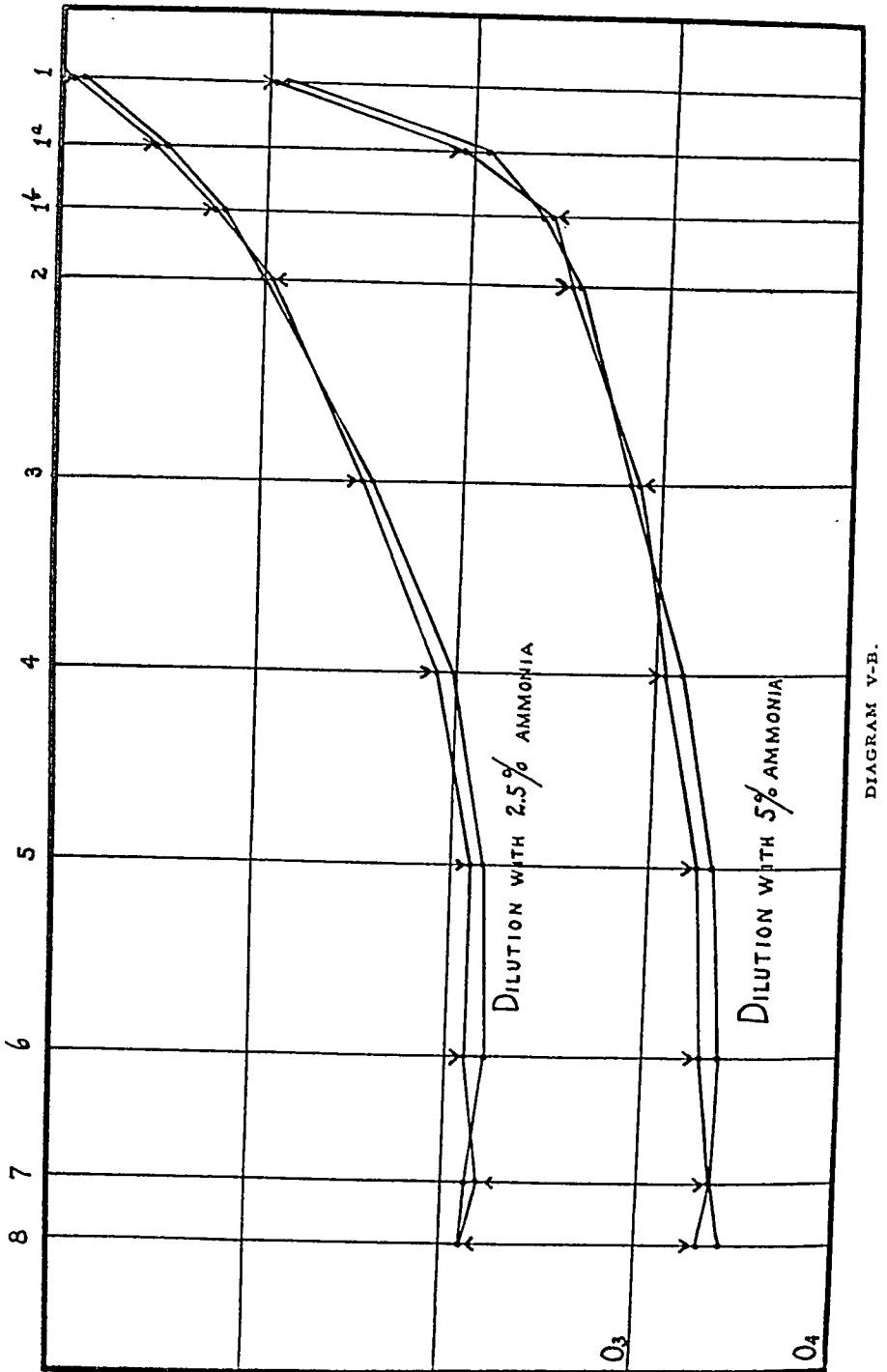


DIAGRAM V-B.

## NOTE ON THE CONCENTRATION OF DIPHTHERIA TOXIN.

BY P. G. HEINEMANN.

(From the Bacteriological Laboratory of the University of Chicago.)

(Received for publication, May 7, 1908.)

It is well known that a high potency diphtheria toxin is advantageous in the production of diphtheria antitoxin. The antitoxin potency depends to some extent on the injection of increasing doses of toxin. The amount of toxin which can be given to a horse with safety is limited, however, by the volume; as a rule the use of a toxin of less potency than 0.005 is not advantageous. A toxin, the m.l.d. of which lies in the fourth decimal, has been obtained occasionally, but not with regularity. Such powerful toxins would enable us to give higher doses, so that sera of high potency could perhaps be produced. By precipitation we can also remove some of the constituents of the toxin broth, which are useless, if not injurious.

In the literature several methods have been described for preparing a concentrated and purified toxin, mostly, however, in small amounts and under experimental conditions. Thus Brieger and Fraenkel<sup>1</sup> precipitated toxin with ethyl alcohol acidulated with acetic acid. They stated that ammonium sulphate, sodium chloride, and sodium sulphate also precipitate toxins. Wassermann and Proskauer<sup>2</sup> precipitated toxin by addition of eight to ten times the volume of acidulated absolute alcohol. Another method used by these authors consists in evaporation to one-tenth the volume, dialysis to remove the peptones and then precipitation by use of acidulated 60 to 70 per cent alcohol. Brieger and Cohn<sup>3</sup> precipitated tetanus toxin by supersaturation with ammonium sulphate and stated that the precipitability of tetanus toxin with ammonium sulphate

<sup>1</sup> *Berl. klin. Woch.* p. 241, xxvii, 1890.

<sup>2</sup> *Deutsche med. Woch.*, xvii, p. 585, 1891.

<sup>3</sup> *Zeitschr. f. Hyg.*, xv, p. 1, 1893.

decreases as the toxin becomes more free from foreign substances. Brieger<sup>1</sup> then used neutral lead acetate and precipitated the toxin almost quantitatively. The lead acetate is rather difficult to get rid of. This method is better adapted for diphtheria than tetanus toxin. Finally Breiger and Boer<sup>2</sup> prepared a double salt by precipitating the toxin with a 1 per cent zinc chloride solution and removed the zinc by means of ammonium phosphate. The toxin was then purified by redissolving and reprecipitating with ammonium sulphate. Banzhaf<sup>3</sup> has used alcohol as precipitating agent, but later states<sup>4</sup> that the advantages of concentrated toxins do not justify the use, when the expense of preparation is considered. This would also be true as regards the use of some other precipitating agents, as acetone. The experience of other observers gives one the impression that toxins suffer in potency by use of alcohol and acids. Salts, however, do not seem to affect them. Banzhaf also stated that his experiments with horses do not prove that a higher potency is obtained with concentrated toxins than is obtained with ordinary toxins. The chances of abscess formation, however, are probably lessened with a reduction of volume injected and the possibility of saving a weak toxin is an additional advantage.

In preparing a concentrated toxin to immunize horses it is not as essential to get a toxin as nearly chemically pure as possible as to prepare it with small loss and as inexpensively as possible. I have had some success in concentrating diphtheria toxin in a series of experiments by the following method: To a given volume of toxin is added twice the volume of a saturated solution of ammonium sulphate with constant stirring. Less ammonium sulphate solution does not precipitate the toxin completely and more than twice the volume was found to be superfluous. The mixture is allowed to stand for twenty-four hours, after which a brownish-yellow scum forms and sometimes a sediment of the same color. Both the scum and sediment are then collected on a large folded filter. Twenty liters and more

<sup>1</sup> *Zeitschr. f. Hyg.*, xix, p. 101, 1895.

<sup>2</sup> *Deutsche med. Woch.*, xxii, p. 783, 1896.

<sup>3</sup> *Collected Studies from the Research Laboratory of the Department of Health of New York City*, p. 35, 1905.

<sup>4</sup> *Ibid.*, p. 101, 1906.



can be filtered on one filter in a few hours. The precipitate is scraped from the paper before it is entirely dry, since it comes off more readily while moist. The precipitate is then dialyzed in a collodion or parchment sac. Collodion was used exclusively at first because Oppenheimer<sup>1</sup> has stated that part of the toxin is lost by dialyzing through parchment. If heavy parchment is used, however, this does not seem to be true, at least one experiment made seemed to show this. In this experiment the precipitate was divided into two equal parts and one-half dialyzed in a collodion sac, hardened with chloroform and alcohol, and the other half in parchment. The two lots of toxin were of substantially the same potency. Since it is difficult to prepare a collodion sac and seal it to a glass tube without sometimes having a small leak, it may be more advisable to use heavy parchment for dialysis.

Similarly to the precipitate a small portion of the filtrate in each experiment was dialyzed under identical conditions and two to four cubic centimeters injected into guinea pigs. The guinea pigs showed no effect resembling diphtheria poisoning whatever, but steadily gained weight, except in one case, where the animal died in nine days and cultures from the heart's blood showed the presence of staphylococci, but no lesions of diphtheria poisoning.

The toxin solution is removed from the sac after five or six days. It is filtered through paper and then is a clear dark brown fluid. As a preservative I add 0.3 per cent carbolic acid. I found that by adding a larger amount of carbolic acid to the toxin a heavy precipitate forms which seems to contain much of the toxin. Whenever this happens it is advisable to add enough water to dissolve the precipitate although the potency is diminished proportionately.

The following table shows the results of the six experiments made so far:

<sup>1</sup> *Die Toxine und Antitoxine.*

Original m.l. d.	M.l.d. of concentrated toxin.	Theoretical m.l.d.*	Dialyzed through—
0.003	0.0007	0.0004	Collodion.
0.006	0.0005	0.0004	"
0.003	0.0003	0.0002	"
0.009	0.0007	0.0006	"
0.009	0.0007	0.0006	Parchment.
less than 0.005	0.00035	less than 0.00025	Collodion.

\* Theoretical m.l.d. is the m.l.d. which would result if there had been no loss whatever by manipulation.

The table shows that the loss is not great and undoubtedly can be reduced by perfecting the technic. By this method it is possible to make use of a toxin, which is not potent enough to be used without concentration.

# ON THE PART PLAYED BY THE ALKALI IN THE HYDROLYSIS OF PROTEINS BY TRYPSIN.

By T. BRAILSFORD ROBERTSON and C. L. A. SCHMIDT.

(From the Rudolph Spreckels Physiological Laboratory and the Laboratory  
of Physical Chemistry of the University of California.)

(Received for publication, May 15, 1908.)

## I. INTRODUCTION.

The part played by the alkali and acid in protein hydrolysis by enzymes is by no means clear. It is known that the activity of pepsin is greatly enhanced by acid while that of trypsin is greatly enhanced by alkali, and, since acids and alkalies are well-known hydrolyzing agents, the most natural assumption regarding their influence upon protein hydrolysis by enzymes is to suppose that they play the part of accessory catalysors. As regards the influence of acids upon the hydrolysis of proteins by pepsin, however, there are many well-known facts which speak against this view. The influence of acids is by no means proportional to their degree of dissociation as would be expected were their influence due to a catalytic action of the hydrogen ion.<sup>1</sup> On the contrary hydrochloric acid has an almost specific action upon the hydrolysis of proteins by pepsin, so that many observers have inclined to the belief that the real ferment is in this instance a compound of pepsin with hydrochloric acid.<sup>2</sup> As regards the influence of alkalies upon the activity of trypsin the facts, so far as has been hitherto ascertained, are much more in harmony with the view that the alkali acts as an accessory catalysor, since it is stated that the hydroxides of the alkalies and alkaline earths act in proportion to their degree of dissociation,<sup>3</sup> although

<sup>1</sup> Cf. Klug: *Arch. f. d. ges. Physiol.*, lxxv, p. 330, 1896; A. E. Taylor: "On Fermentation," *University of California Publications, Pathology*, 1907, p. 249; Berg and Gies: *This Journal*, ii, p. 489, 1907.

<sup>2</sup> C. Schmidt: *Ann. d. Chem.*, lxi, p. 311, 1847.

<sup>3</sup> A. E. Taylor: *Loc. cit.*, p. 251.

divergencies from the proportionality of the action to the degree of dissociation have been found.<sup>1</sup>

It has been frequently pointed out that the progress of the hydrolysis of proteins is accompanied by marked changes in the acidity or alkalinity of the solutions in which the hydrolysis occurs<sup>2</sup> and it has been suggested that this phenomenon is to be attributed to the fact that proteins split up, during hydrolysis, into amino-acids, so that where one molecule of amino-acid (the protein itself) initially existed in the solution and partially neutralized the acids or bases present, after the digestion has proceeded for some time, several molecules now exist, each of which neutralizes as much or nearly as much acid or base as the original molecule—hence any excess of acid or base tends to disappear during the hydrolysis and the system tends to approach neutrality.<sup>3</sup>

Since the concentration of hydrogen and hydroxyl ions changes markedly during the progress of hydrolysis, we would expect this fact to exert a marked influence upon the progress of the reaction. It happens, however, that the majority of the hitherto published investigations upon the progress of protein hydrolysis by trypsin have been conducted under such conditions that the alkalinity of the system remained practically constant throughout the experiment. Thus Bayliss' experiments were conducted at a high alkalinity (i. e., 2 cc.  $\frac{N}{3}$  added to 6 cc. of the system) and any changes due to the alkali-binding power of the products must have been negligible in comparison with the total concentration of alkali.<sup>4</sup>

Robertson's experiments were conducted in neutral solutions in which no perceptible change in  $H^+$  or  $OH^-$  concentration occurs during the progress of hydrolysis.<sup>5</sup> Henri and his pupils also

<sup>1</sup> Berg and Gies: *Loc. cit.*

<sup>2</sup> Gillespie: *Journal of Anatomy and Physiology*, xxvii, p. 195, 1893; A. E. Taylor: *Univ. of California Publications, Pathology*, i, p. 7, 1904; Greenwood and Saunders: *Journal of Physiology*, xvi, p. 441, 1894; T. Brailsford Robertson: *This Journal*, ii, p. 368, 1907.

<sup>3</sup> T. Brailsford Robertson: *Loc. cit.*

<sup>4</sup> Bayliss: *Arch. des science biol.* (St. Petersburg), xi, p. 261, 1904, reprinted in the collected papers of the University College Physiological Laboratory, London, xiii.

<sup>5</sup> T. Brailsford Robertson: *Loc. cit.*

worked with neutral solutions. (Gelatin and neutral caseinates.)<sup>1</sup>

All these observers obtained results in excellent agreement with the formula

$$k = \frac{1}{t} \log. \frac{a}{a-x}$$

indicating that the hydrolysis follows closely the course of a monomolecular reaction. In Taylor's experiment<sup>2</sup> the alkalinity of the system changed markedly with the progress of hydrolysis, but, as our own experiments show, the influence of the alkali is of such a nature that its decrease in concentration would not, under the conditions of Taylor's experiments, have much effect upon the progress of the hydrolysis, and in fact Taylor's results also confirm the monomolecular formula.

The above quoted investigation by Taylor shows clearly that the alkali, in the hydrolysis of protein by trypsin, does not play the part of an accessory catalysor, for if this were the case, since a considerable portion of the alkali disappeared during hydrolysis, the monomolecular formula could not have been obtained. Nevertheless our own investigations were initiated in the belief that the alkali acted as an accessory catalysor and that, therefore, in cases where the alkali diminishes appreciably during the reaction the curve of the hydrolysis would be found to obey the formula representing the progress of an auto catalyzed reaction in which the catalysor is consumed as the reaction proceeds; that is, the formula,

$$k(a-b)t = \log. \frac{b(a-x)}{a(b-x)}$$

where  $a$  and  $b$  are, respectively, the initial concentration of the substrate and of the catalysor. Somewhat to our surprise, however, we found, at an early stage in the investigations, that the course of the hydrolysis is by no means represented at all stages by this formula nor by any probable modification of the formula which suggested itself, and our results are such as to

<sup>1</sup> Henri and Des Bancel: *Compt. rend. de l'Acad. des sci.*, cxxxvi, p. 1581, 1903; *Compt. rend. soc. biol.*, lv, pp. 563, 787 and 789, 1903.

<sup>2</sup> A. E. Taylor: *Univ. of Calif. Publ. Pathol.*, i, p. 7, 1904.

indicate that the part played by the hydroxyl ions is somewhat more complex than that of an accessory catalysor.

## II. EXPERIMENTAL.

The method employed was to follow the changes in  $\text{OH}^-$  concentration during the progress of the hydrolysis with the aid of the gas chain. The apparatus employed was similar to that used by one of us in the determination of the potential of a hydrogen electrode in acid and alkaline borate solutions<sup>1</sup> and is fully described in the paper referred to. The hydrogen was generated by the electrolysis of water, and subsequently passed over platinized asbestos heated sufficiently to rid it of any hydrogen peroxide or oxygen. The hydrogen then passed through the very fine meshes of a cylinder of platinized platinum gauze which formed the electrode. In this way the three phases, platinum, hydrogen and solution were brought into most intimate contact and equilibrium was quickly established. The other extremity of the chain was formed by a standard calomel electrode using  $\frac{N}{10}$  KCl.<sup>2</sup> Connection between the calomel electrode and the cell containing the protein solution was made through two small beakers filled with  $\frac{N}{10}$  KCl, the second beaker being connected with the protein solution by means of a U-tube filled with agar-agar which had been made up in a saturated solution of KCl. The cell containing the solution under investigation had a side cup attached into which the U-tube filled with agar-agar was dipped, thus preventing contamination of the main body of the solution by KCl. Any contact differences of potential in the system due to the fact that KCl was not introduced into the solution under investigation were eliminated by the interpolation of the saturated KCl.<sup>3</sup> The remainder of the apparatus was fitted up in the usual manner, a sensitive galvanometer being, however, used as the zero instrument instead of a capillary electrometer.

The experiments were conducted upon the hydrolysis of casein and of protamine. The casein was Eimer and Amend's C. P.

<sup>1</sup> C. L. A. Schmidt and C. P. Finger: *Journal of Physical Chem.* (about to appear).

<sup>2</sup> Richards: *Zeitschr. f. physik. Chem.*, xxiv, p. 37, 1897.

<sup>3</sup> Bjerrum: *Ibid.*, liii, p. 428, 1905.

preparation "nach Hammarsten;" for the purpose of this investigation it was further purified by the method described by Robertson.<sup>1</sup> It was then shaken up with the various concentrations of NaOH until no more would dissolve at 25° C. The solution was then filtered through glass wool and filter paper. Most of the filter papers in the laboratories were found to be slightly acid, but this source of contamination was eliminated by rejecting the first portion of the filtrate. In this way solutions of neutral sodium caseinate were obtained.<sup>2</sup>

The protamine used had been prepared from salmon sperm by Dr. A. E. Taylor and was very kindly supplied to us by the Department of Pathology. It was in the form of the sulphate. This was directly dissolved in distilled water in approximately the concentration desired. In both sets of experiments the procedure was as follows: The requisite amount of Grüber's pancreatin (nach Spateholz)<sup>3</sup> was dissolved in dilute alkali and the solution filtered through glass wool and immediately added to the casein or protamine solution under investigation. The mixture thus prepared was then placed in the cell and hydrogen allowed to bubble through at a slow rate. To all protein solutions toluol was added to prevent bacterial infection, and a layer of toluol was, in addition, placed upon the surface of the mixture in the cell to further secure freedom from bacterial infection and to prevent foaming. A previous determination with a borate mixture showed that the toluol has no influence upon the equilibrium between the electrode and the solution. Hydrogen was passed through at a slow rate and after about half an hour the first reading was taken.

In alkaline solutions, when hydrogen is first passed through the electrode and solution, the solution pressure of the hydrogen in the electrode is considerably greater than the osmotic pressure of the hydrogen ions in the solution—hence as the system approaches equilibrium, the apparent alkalinity, as indicated by the instrument, progressively increases; on the other hand, the real alkalinity of the solution under investigation is progressively decreasing on account of hydrolysis. The curves representing

<sup>1</sup> T. Brailsford Robertson: *This Journal*, ii, p. 317, 1907.

<sup>2</sup> T. Brailsford Robertson: *Loc. cit.*

<sup>3</sup> Cf. A. E. Taylor: *Univ. of Calif. Publ. Pathol.*, i, p. 7, 1904.

these two processes will evidently meet at some point and equilibrium between the electrode and the solution will be established more rapidly than if hydrolysis were not taking place. From the point of intersection of these two curves, the potential of the electrode simply follows the decrease of alkalinity of the solution, since any displacement in equilibrium is rectified by the changes occurring in the solution and the velocity of hydrolysis was, in all our experiments, low compared with the velocity with which the electrode came to equilibrium except, possibly, in the initial stages where, however, the changes in the solution are as explained above, aiding the electrode in attaining equilibrium.

The experiments were carried out at the temperature of the laboratory (average  $16^{\circ}$  C. ) which, while fairly constant for periods of one or two hours, probably varied over an extreme range of  $3^{\circ}$  C. to  $4^{\circ}$  C. throughout the day. The storage battery was checked against the standard Weston cell after nearly every reading.

All the sodium hydrate solutions employed were only of approximate strength and the concentration of the protein and of the trypsin employed were likewise approximate.

The following is a tabular representation of our experimental results: In the first column is given the time in minutes after mixing the trypsin and alkali with the solution of protein. In the second column is given the time  $(t - t_1)$  in minutes after the electrode has come to equilibrium with the solution; this is indicated as explained above, by the occurrence of a maximum in the potential. Since the exact position of this maximum might readily escape detection in the readings, the time of the first reading subsequent to the maximum was taken to represent the time at which the electrode had come to equilibrium with the solution. In the third column is given the observed E.M.F. in volts, in the fourth is given the  $\text{OH}^-$  concentration corresponding to these voltages,<sup>1</sup> in the fifth is given the change  $(x)$  in hydroxyl concentration since the first reading at which the electrode had come to equilibrium with the solution.

<sup>1</sup> The  $\text{H}^+$  concentration at the neutral point was taken as  $.75 \times 10^{-7}$ , and the  $\text{OH}^-$  concentrations were calculated from the Nernst formula:

$$\pi = \frac{1.99}{n} \frac{T \times 10^{-4}}{c_1} \cdot \log \frac{c_1}{c_2}$$



TABLE I.

EXPERIMENT 1. CASEIN.  $\frac{N}{200}$  NaOH "saturated" with casein to which was added an equal volume of  $\frac{N}{300}$  NaOH. 10 milligrams of trypsin in 50 cc. Temperature, 19°.

Time in minutes after mixing.	$t-t_1$ .	E.M.F. in volts.	$C_{OH^-}$ .	$x$ .	$k$ (Monomolecular).	$k$ (Bimolecular).
34		0.798	$5.95 \times 10^{-7}$			
38	0	0.796	$5.50 \times 10^{-7}$	0		
51	13	0.794	$5.06 \times 10^{-7}$	$0.44 \times 10^{-7}$	$269 \times 10^{-5}$	$118 \times 10^{-5}$
65	27	0.791	$4.50 \times 10^{-7}$	$1.00 \times 10^{-7}$	$316 \times 10^{-5}$	$146 \times 10^{-5}$
88	50	0.788	$4.00 \times 10^{-7}$	$1.50 \times 10^{-7}$	$275 \times 10^{-5}$	$136 \times 10^{-5}$
126	88	0.784	$3.40 \times 10^{-7}$	$2.10 \times 10^{-7}$	$236 \times 10^{-5}$	$127 \times 10^{-5}$
158	120	0.782	$3.15 \times 10^{-7}$	$2.35 \times 10^{-7}$	$201 \times 10^{-5}$	$113 \times 10^{-5}$
182	144	0.779	$2.79 \times 10^{-7}$	$2.71 \times 10^{-7}$	$204 \times 10^{-5}$	$123 \times 10^{-5}$
209	171	0.777	$2.56 \times 10^{-7}$	$2.94 \times 10^{-7}$	$194 \times 10^{-5}$	$123 \times 10^{-5}$
215	177	0.775	$2.38 \times 10^{-7}$	$3.12 \times 10^{-7}$	$205 \times 10^{-5}$	$134 \times 10^{-5}$
247	209	0.774	$2.30 \times 10^{-7}$	$3.20 \times 10^{-7}$	$181 \times 10^{-5}$	$121 \times 10^{-5}$
347	309	0.770	$1.96 \times 10^{-7}$	$3.54 \times 10^{-7}$	$145 \times 10^{-5}$	$107 \times 10^{-5}$
410	372	0.767	$1.72 \times 10^{-7}$	$3.78 \times 10^{-7}$	$135 \times 10^{-5}$	$107 \times 10^{-5}$
530	492	0.766	$1.66 \times 10^{-7}$	$3.84 \times 10^{-7}$	$106 \times 10^{-5}$	$86 \times 10^{-5}$
652	614	0.763	$1.47 \times 10^{-7}$	$4.03 \times 10^{-7}$	$93 \times 10^{-5}$	$81 \times 10^{-5}$

We may reasonably assume that the change in  $OH^-$  concentration is proportional to the amount of substrate which has undergone hydrolysis. Since at the end of the experiment, nearly all the hydroxyl is neutralized, we may assume that at equilibrium the solution would be neutral. If, therefore, the hydrolysis followed the course of a monomolecular reaction, it should obey the formula:

$$k(t - t_1) = \log \frac{\beta}{\beta - x}$$

where  $\beta$  is the concentration of the  $OH^-$  ions at the time ( $t_1$ ) when the electrode first comes into equilibrium with the solution. On the other hand, if it followed the course of a bimolecular reaction, it should obey the formula:

$$k(t - t_1) = \frac{x}{\beta(\beta - x)}$$

where  $\beta$  and  $t_1$  have the same significance as before. In the sixth column of the tables are given the constants calculated upon the assumption that the reaction obeys the monomolecular formula, while in the seventh are given the constants calculated upon the assumption that the reaction obeys the bimolecular formula.

In the tenth and eleventh experiments (with protamine: cf. Tables X and XI) the reaction was allowed to proceed to a certain point and then additional alkali was added to the solution in the cell and the mixture shaken. As at the beginning of the experiment, the indicated potential at first rises and then falls after this procedure. The first reading after the maximum

TABLE II.

EXPERIMENT 2. CASEIN.  $\frac{N}{100}$  NaOH "saturated" with casein to which was added an equal volume of  $\frac{N}{300}$  NaOH. 10 milligrams of trypsin in 50 cc. Temperature  $15^\circ$  to  $18^\circ$ .

Time in minutes after mixing.	$t-t_1$ .	E.M.F. in volts.	$C_{OH^-}$ .	$x$	$k$ (Monomolecular).	$k$ (Bimolecular).
51		0.837	$28.4 \times 10^{-7}$			
59	0	0.836	$27.3 \times 10^{-7}$	0		
69	10	0.835	$26.2 \times 10^{-7}$	$1.1 \times 10^{-7}$	$180 \times 10^{-5}$	$154 \times 10^{-6}$
73	14	0.833	$24.2 \times 10^{-7}$	$3.1 \times 10^{-7}$	$375 \times 10^{-5}$	$336 \times 10^{-6}$
99	40	0.829	$20.6 \times 10^{-7}$	$6.7 \times 10^{-7}$	$305 \times 10^{-5}$	$296 \times 10^{-6}$
126	67	0.823	$16.2 \times 10^{-7}$	$11.1 \times 10^{-7}$	$338 \times 10^{-5}$	$376 \times 10^{-6}$
153	94	0.817	$12.8 \times 10^{-7}$	$14.5 \times 10^{-7}$	$352 \times 10^{-5}$	$445 \times 10^{-6}$
197	138	0.809	$9.3 \times 10^{-7}$	$18.0 \times 10^{-7}$	$340 \times 10^{-5}$	$516 \times 10^{-6}$
225	166	0.804	$7.6 \times 10^{-7}$	$19.7 \times 10^{-7}$	$336 \times 10^{-5}$	$574 \times 10^{-6}$
246	187	0.801	$6.7 \times 10^{-7*}$	$20.6 \times 10^{-7}$	$326 \times 10^{-5}$	$600 \times 10^{-6}$
276	217	0.798	$5.95 \times 10^{-7}$	$21.35 \times 10^{-7}$	$305 \times 10^{-5}$	$604 \times 10^{-6}$
303	244	0.795	$5.45 \times 10^{-7}$	$21.85 \times 10^{-7}$	$293 \times 10^{-5}$	$627 \times 10^{-6}$
338	279	0.792	$4.7 \times 10^{-7}$	$22.6 \times 10^{-7}$	$275 \times 10^{-5}$	$635 \times 10^{-6}$
368	309	0.788	$4.0 \times 10^{-7}$	$23.3 \times 10^{-7}$	$270 \times 10^{-5}$	$690 \times 10^{-6}$
408	349	0.786	$3.7 \times 10^{-7}$	$23.6 \times 10^{-7}$	$250 \times 10^{-5}$	$670 \times 10^{-6}$
443	384	0.785	$3.5 \times 10^{-7}$	$23.8 \times 10^{-7}$	$232 \times 10^{-5}$	$642 \times 10^{-6}$
470	411	0.783	$3.3 \times 10^{-7}$	$24.0 \times 10^{-7}$	$224 \times 10^{-5}$	$654 \times 10^{-6}$
547	488	0.779	$2.8 \times 10^{-7}$	$24.5 \times 10^{-7}$	$203 \times 10^{-5}$	$660 \times 10^{-6}$
601	542	0.777	$2.6 \times 10^{-7}$	$24.7 \times 10^{-7}$	$190 \times 10^{-5}$	$656 \times 10^{-6}$
647	588	0.776	$2.5 \times 10^{-7}$	$24.8 \times 10^{-7}$	$178 \times 10^{-5}$	$627 \times 10^{-6}$
722	663	0.776	$2.5 \times 10^{-7}$	$24.8 \times 10^{-7}$	$157 \times 10^{-5}$	$556 \times 10^{-6}$

TABLE III.

EXPERIMENT 3. CASEIN.  $\frac{N}{200}$  NaOH "saturated" with casein to which was added an equal volume of  $\frac{N}{100}$  NaOH. 10 milligrams of trypsin in 50 cc.

Time in minutes after mixing.	$t-t_1$	E.M.F. in volts.	$C_{OH^-}$	$x$	$k$ (Monomolecular).	$k$ (Bimolecular).
41		0.790	$4.35 \times 10^{-7}$			
56	0	0.790	$4.35 \times 10^{-7}$	0		
96	40	0.785	$3.54 \times 10^{-7}$	$0.81 \times 10^{-7}$	$230 \times 10^{-5}$	$132 \times 10^{-5}$
112	56	0.783	$3.28 \times 10^{-7}$	$1.07 \times 10^{-7}$	$218 \times 10^{-5}$	$134 \times 10^{-5}$
133	77	0.779	$2.79 \times 10^{-7}$	$1.56 \times 10^{-7}$	$251 \times 10^{-5}$	$167 \times 10^{-5}$
188	132	0.777	$2.56 \times 10^{-7}$	$1.79 \times 10^{-7}$	$175 \times 10^{-5}$	$122 \times 10^{-5}$
205	149	0.772	$2.08 \times 10^{-7}$	$2.27 \times 10^{-7}$	$215 \times 10^{-5}$	$167 \times 10^{-5}$
237	181	0.770	$1.96 \times 10^{-7}$	$2.39 \times 10^{-7}$	$191 \times 10^{-5}$	$155 \times 10^{-5}$
271	215	0.769	$1.83 \times 10^{-7}$	$2.52 \times 10^{-7}$	$175 \times 10^{-5}$	$147 \times 10^{-5}$
305	249	0.766	$1.64 \times 10^{-7}$	$2.71 \times 10^{-7}$	$170 \times 10^{-5}$	$153 \times 10^{-5}$
332	276	0.765	$1.57 \times 10^{-7}$	$2.78 \times 10^{-7}$	$160 \times 10^{-5}$	$147 \times 10^{-5}$
388	332	0.764	$1.49 \times 10^{-7}$	$2.86 \times 10^{-7}$	$140 \times 10^{-5}$	$133 \times 10^{-5}$
486	430	0.762	$1.40 \times 10^{-7}$	$2.95 \times 10^{-7}$	$115 \times 10^{-5}$	$113 \times 10^{-5}$
626	570	0.759	$1.24 \times 10^{-7}$	$3.11 \times 10^{-7}$	$96 \times 10^{-5}$	$102 \times 10^{-5}$

TABLE IV.

EXPERIMENT 4. CASEIN.  $\frac{N}{100}$  NaOH "saturated" with casein to which was added an equal volume of  $\frac{N}{300}$  NaOH. 20 milligrams of trypsin in 50 cc. Temperature  $12^\circ$  to  $14^\circ$ .

Time in minutes after mixing.	$t-t_1$	E.M.F. in volts.	$C_{OH^-}$	$x$	$k$ (Monomolecular).	$k$ (Bimolecular).
33		0.838	$29.6 \times 10^{-7}$			
44	0	0.836	$27.2 \times 10^{-7}$	0		
61	17	0.831	$22.4 \times 10^{-7}$	$4.8 \times 10^{-7}$	$514 \times 10^{-5}$	$483 \times 10^{-5}$
97	53	0.822	$15.6 \times 10^{-7}$	$11.6 \times 10^{-7}$	$461 \times 10^{-5}$	$518 \times 10^{-5}$
121	77	0.816	$12.3 \times 10^{-7}$	$14.9 \times 10^{-7}$	$452 \times 10^{-5}$	$585 \times 10^{-5}$
181	137	0.804	$7.6 \times 10^{-7}$	$19.6 \times 10^{-7}$	$407 \times 10^{-5}$	$695 \times 10^{-5}$
228	184	0.797	$5.75 \times 10^{-7}$	$21.45 \times 10^{-7}$	$368 \times 10^{-5}$	$747 \times 10^{-5}$
260	216	0.793	$4.9 \times 10^{-7}$	$22.3 \times 10^{-7}$	$346 \times 10^{-5}$	$775 \times 10^{-5}$
285	241	0.791	$4.5 \times 10^{-7}$	$22.7 \times 10^{-7}$	$325 \times 10^{-5}$	$769 \times 10^{-5}$
315	271	0.789	$4.2 \times 10^{-7}$	$23.0 \times 10^{-7}$	$301 \times 10^{-5}$	$750 \times 10^{-5}$
345	301	0.786	$3.7 \times 10^{-7}$	$23.5 \times 10^{-7}$	$288 \times 10^{-5}$	$776 \times 10^{-5}$
387	343	0.784	$3.4 \times 10^{-7}$	$23.8 \times 10^{-7}$	$264 \times 10^{-5}$	$750 \times 10^{-5}$
431	387	0.782	$3.15 \times 10^{-7}$	$24.05 \times 10^{-7}$	$243 \times 10^{-5}$	$725 \times 10^{-5}$

TABLE V.

EXPERIMENT 5. CASEIN.  $\frac{N}{100}$  NaOH "saturated" with casein to which was added an equal volume of  $\frac{N}{100}$  NaOH. 10 milligrams of trypsin in 50 cc. Temperature  $12^{\circ}$  to  $15^{\circ}$ .

Time in minutes after mixing.	$t-t_1$ .	E.M.F. in volts.	$C_{OH^-}$	$x$ .	$k$ (Monomolecular).	$k$ (Bimolecular).
38		0.849	$46.0 \times 10^{-7}$			
57		0.853	$54.0 \times 10^{-7}$			
132	0	0.847	$42.4 \times 10^{-7}$	0		
160	28	0.842	$34.6 \times 10^{-7}$	$7.8 \times 10^{-7}$	$315 \times 10^{-5}$	$189 \times 10^{-6}$
197	65	0.834	$25.2 \times 10^{-7}$	$17.2 \times 10^{-7}$	$347 \times 10^{-5}$	$248 \times 10^{-6}$
216	84	0.831	$22.4 \times 10^{-7}$	$20.0 \times 10^{-7}$	$330 \times 10^{-5}$	$251 \times 10^{-6}$
243	111	0.827	$19.0 \times 10^{-7}$	$23.4 \times 10^{-7}$	$316 \times 10^{-5}$	$262 \times 10^{-6}$
264	132	0.823	$16.2 \times 10^{-7}$	$26.2 \times 10^{-7}$	$317 \times 10^{-5}$	$289 \times 10^{-6}$
284	152	0.821	$15.0 \times 10^{-7}$	$27.4 \times 10^{-7}$	$297 \times 10^{-5}$	$284 \times 10^{-6}$
299	167	0.818	$12.85 \times 10^{-7}$	$29.55 \times 10^{-7}$	$310 \times 10^{-5}$	$326 \times 10^{-6}$
322	190	0.815	$11.8 \times 10^{-7}$	$30.6 \times 10^{-7}$	$293 \times 10^{-5}$	$322 \times 10^{-6}$
359	227	0.809	$9.3 \times 10^{-7*}$	$33.1 \times 10^{-7}$	$290 \times 10^{-5}$	$370 \times 10^{-6}$
397	265	0.805	$7.9 \times 10^{-7}$	$34.5 \times 10^{-7}$	$275 \times 10^{-5}$	$389 \times 10^{-6}$
509	377	0.797	$5.75 \times 10^{-7}$	$36.65 \times 10^{-7}$	$230 \times 10^{-5}$	$390 \times 10^{-6}$
581	449	0.791	$4.5 \times 10^{-7}$	$37.9 \times 10^{-7}$	$217 \times 10^{-5}$	$443 \times 10^{-6}$
1301	1169	0.772	$2.1 \times 10^{-7}$	$40.3 \times 10^{-7}$	$112 \times 10^{-5}$	$404 \times 10^{-6}$

TABLE VI.

EXPERIMENT 6. PROTAMIN. 2 grams of protamin sulphate to 1 litre of solution. 10 milligrams of trypsin and 1.4 cc. of  $\frac{N}{10}$  NaOH added to 50 cc.

Time in minutes after mixing.	$t-t_1$ .	E.M.F. in volts.	$C_{OH^-}$	$x$ .	$k$ (Monomolecular).	$k$ (Bimolecular).
58		0.819	$13.8 \times 10^{-7}$			
76	0	0.815	$11.8 \times 10^{-7}$	0		
98	22	0.810	$9.7 \times 10^{-7}$	$2.1 \times 10^{-7}$	$393 \times 10^{-5}$	$83 \times 10^{-5}$
128	52	0.803	$7.3 \times 10^{-7}$	$4.5 \times 10^{-7}$	$403 \times 10^{-5}$	$104 \times 10^{-5}$
169	93	0.796	$5.6 \times 10^{-7}$	$6.2 \times 10^{-7}$	$349 \times 10^{-5}$	$101 \times 10^{-5}$
193	117	0.793	$4.9 \times 10^{-7}$	$6.9 \times 10^{-7}$	$327 \times 10^{-5}$	$102 \times 10^{-5}$
217	141	0.790	$4.3 \times 10^{-7}$	$7.5 \times 10^{-7}$	$310 \times 10^{-5}$	$105 \times 10^{-5}$
242	166	0.787	$3.85 \times 10^{-7}$	$7.95 \times 10^{-7}$	$293 \times 10^{-5}$	$105 \times 10^{-5}$
271	195	0.785	$3.55 \times 10^{-7}$	$8.25 \times 10^{-7}$	$267 \times 10^{-5}$	$101 \times 10^{-5}$
307	231	0.783	$3.3 \times 10^{-7}$	$8.5 \times 10^{-7}$	$240 \times 10^{-5}$	$95 \times 10^{-5}$
410	334	0.780	$2.9 \times 10^{-7}$	$8.9 \times 10^{-7}$	$182 \times 10^{-5}$	$78 \times 10^{-5}$
590	514	0.777	$2.6 \times 10^{-7}$	$9.2 \times 10^{-7}$	$128 \times 10^{-5}$	$58 \times 10^{-5}$

TABLE VII.

EXPERIMENT 7. PROTAMIN. 2 grams of protamin sulphate in 1 litre of solution. 10 milligrams of trypsin and 1.5 cc. of  $\frac{N}{10}$  NaOH to 50 cc.

Time in minutes after mixing.	$t-t_1$ .	E.M.F. in volts.	$C_{OH^-}$ .	$x$ .	$k$ (Monomolecular).	$k$ (Bimolecular).
23		0.847	$42.5 \times 10^{-7}$			
35	0	0.843	$36.2 \times 10^{-7}$	0		
54	19	0.840	$32.1 \times 10^{-7}$	$4.1 \times 10^{-7}$	$279 \times 10^{-5}$	$185 \times 10^{-6}$
76	41	0.836	$27.3 \times 10^{-7}$	$8.9 \times 10^{-7}$	$298 \times 10^{-5}$	$220 \times 10^{-6}$
118	83	0.831	$22.4 \times 10^{-7}$	$13.8 \times 10^{-7}$	$253 \times 10^{-5}$	$205 \times 10^{-6}$
167	132	0.824	$16.9 \times 10^{-7}$	$19.3 \times 10^{-7}$	$250 \times 10^{-5}$	$239 \times 10^{-6}$
206	171	0.819	$13.85 \times 10^{-7}$	$22.35 \times 10^{-7}$	$245 \times 10^{-5}$	$260 \times 10^{-6}$
240	205	0.817	$12.8 \times 10^{-7}$ *	$23.4 \times 10^{-7}$	$220 \times 10^{-5}$	$246 \times 10^{-6}$
270	235	0.815	$11.8 \times 10^{-7}$	$24.4 \times 10^{-7}$	$207 \times 10^{-5}$	$243 \times 10^{-6}$
309	274	0.813	$10.9 \times 10^{-7}$	$25.3 \times 10^{-7}$	$190 \times 10^{-5}$	$234 \times 10^{-6}$
355	320	0.812	$10.5 \times 10^{-7}$	$25.7 \times 10^{-7}$	$168 \times 10^{-5}$	$211 \times 10^{-6}$
415	380	0.809	$9.3 \times 10^{-7}$	$26.9 \times 10^{-7}$	$156 \times 10^{-5}$	$212 \times 10^{-6}$
460	425	0.809	$9.3 \times 10^{-7}$	$26.9 \times 10^{-7}$	$139 \times 10^{-5}$	$190 \times 10^{-6}$

TABLE VIII.

EXPERIMENT 8. PROTAMIN. 10 grams of protamin sulphate in 1 litre of solution. 10 milligrams of trypsin and 2 cc. of  $\frac{N}{10}$  NaOH to 25 cc.

Time in minutes after mixing.	$t-t_1$ .	E.M.F. in volts.	$C_{OH^-}$ .	$x$ .	$k$ (Monomolecular).	$k$ (Bimolecular).
49		0.799	$6.2 \times 10^{-7}$			
67	0	0.796	$5.6 \times 10^{-7}$	0		
87	20	0.790	$4.3 \times 10^{-7}$	$1.3 \times 10^{-7}$	$578 \times 10^{-5}$	$270 \times 10^{-6}$
101	34	0.787	$3.85 \times 10^{-7}$	$1.75 \times 10^{-7}$	$479 \times 10^{-5}$	$238 \times 10^{-6}$
125	58	0.782	$3.15 \times 10^{-7}$	$2.45 \times 10^{-7}$	$432 \times 10^{-5}$	$239 \times 10^{-6}$
154	87	0.777	$2.6 \times 10^{-7}$	$3.0 \times 10^{-7}$	$382 \times 10^{-5}$	$237 \times 10^{-6}$
210	143	0.771	$2.0 \times 10^{-7}$	$3.6 \times 10^{-7}$	$313 \times 10^{-5}$	$227 \times 10^{-6}$
254	187	0.768	$1.8 \times 10^{-7}$	$3.8 \times 10^{-7}$	$263 \times 10^{-5}$	$202 \times 10^{-6}$
303	236	0.765	$1.6 \times 10^{-7}$	$4.0 \times 10^{-7}$	$231 \times 10^{-5}$	$189 \times 10^{-6}$
358	291	0.763	$1.5 \times 10^{-7}$	$4.1 \times 10^{-7}$	$196 \times 10^{-5}$	$168 \times 10^{-6}$
422	355	0.761	$1.4 \times 10^{-7}$	$4.2 \times 10^{-7}$	$170 \times 10^{-5}$	$151 \times 10^{-6}$

TABLE IX.

EXPERIMENT 9. PROTAMIN. 10 grams of protamin sulphate in 1 litre of solution. 10 milligrams of trypsin and 2.5 cc. of  $\frac{N}{10}$  NaOH to 25 cc.

Time in minutes after mixing.	$t-t_1$ .	E.M.F. in volts.	$C_{OH^-}$ .	$\alpha$ .	$k$ (Monomolecular).	$k$ (Bimolecular).
60		0.837	$28.5 \times 10^{-7}$			
85	0	0.833	$24.3 \times 10^{-7}$	0		
108	23	0.829	$20.7 \times 10^{-7}$	$3.6 \times 10^{-7}$	$305 \times 10^{-5}$	$311 \times 10^{-5}$
136	51	0.821	$15.0 \times 10^{-7}$	$9.3 \times 10^{-7}$	$411 \times 10^{-5}$	$500 \times 10^{-5}$
181	96	0.812	$10.5 \times 10^{-7}$	$13.8 \times 10^{-7}$	$380 \times 10^{-5}$	$564 \times 10^{-5}$
241	156	0.802	$7.0 \times 10^{-7*}$	$17.3 \times 10^{-7}$	$346 \times 10^{-5}$	$647 \times 10^{-5}$
307	222	0.795	$5.3 \times 10^{-7}$	$19.0 \times 10^{-7}$	$298 \times 10^{-5}$	$664 \times 10^{-5}$
356	271	0.791	$4.5 \times 10^{-7}$	$19.8 \times 10^{-7}$	$270 \times 10^{-5}$	$668 \times 10^{-5}$
400	315	0.788	$4.0 \times 10^{-7}$	$20.3 \times 10^{-7}$	$249 \times 10^{-5}$	$664 \times 10^{-5}$
453	368	0.785	$3.5 \times 10^{-7}$	$20.8 \times 10^{-7}$	$227 \times 10^{-5}$	$663 \times 10^{-5}$
502	417	0.782	$3.1 \times 10^{-7}$	$21.2 \times 10^{-7}$	$213 \times 10^{-5}$	$676 \times 10^{-5}$

TABLE X.

EXPERIMENT 10. PROTAMIN. 10 grams of protamin sulphate in 1 litre of solution. 10 milligrams of trypsin and 2 cc. of  $\frac{N}{10}$  NaOH to 25 cc.

Time in minutes after mixing.	$t-t_1$ .	E.M.F. in volts.	$C_{OH^-}$ .	$\alpha$ .	$k$ (Monomolecular).	$k$ (Bimolecular).
33		0.825				
43	0	0.822	$15.6 \times 10^{-7}$	0		
60	17	0.816	$12.3 \times 10^{-7}$	$3.3 \times 10^{-7}$	$611 \times 10^{-5}$	$101 \times 10^{-5}$
76	33	0.811	$10.05 \times 10^{-7}$	$5.45 \times 10^{-7}$	$577 \times 10^{-5}$	$106 \times 10^{-5}$
90	47	0.806	$8.2 \times 10^{-7*}$	$7.4 \times 10^{-7}$	$596 \times 10^{-5}$	$123 \times 10^{-5}$
102	59	0.803	$7.3 \times 10^{-7}$	$8.3 \times 10^{-7}$	$560 \times 10^{-5}$	$124 \times 10^{-5}$
113	70	0.800	$6.6 \times 10^{-7}$	$9.0 \times 10^{-7}$	$533 \times 10^{-5}$	$125 \times 10^{-5}$
19		0.834	$25.3 \times 10^{-7}$			
29	0	0.833	$24.3 \times 10^{-7}$	0		
42	13	0.831	$22.4 \times 10^{-7}$	$1.9 \times 10^{-7}$	$273 \times 10^{-5}$	$27 \times 10^{-5}$
59	30	0.827	$17.8 \times 10^{-7}$	$6.5 \times 10^{-7}$	$450 \times 10^{-5}$	$50 \times 10^{-5}$
80	51	0.824	$16.9 \times 10^{-7}$	$7.4 \times 10^{-7}$	$311 \times 10^{-5}$	$35 \times 10^{-5}$
104	75	0.821	$15.0 \times 10^{-7}$	$9.3 \times 10^{-7}$	$279 \times 10^{-5}$	$34 \times 10^{-5}$
121	92	0.817	$12.8 \times 10^{-7*}$	$11.5 \times 10^{-7}$	$303 \times 10^{-5}$	$40 \times 10^{-5}$
138	109	0.815	$11.8 \times 10^{-7}$	$12.5 \times 10^{-7}$	$288 \times 10^{-5}$	$40 \times 10^{-5}$
162	133	0.812	$10.5 \times 10^{-7}$	$13.8 \times 10^{-7}$	$275 \times 10^{-5}$	$41 \times 10^{-5}$
202	173	0.808	$8.9 \times 10^{-7}$	$15.4 \times 10^{-7}$	$252 \times 10^{-5}$	$41 \times 10^{-5}$
277	248	0.801	$6.7 \times 10^{-7}$	$17.6 \times 10^{-7}$	$226 \times 10^{-5}$	$41 \times 10^{-5}$

TABLE XI.

EXPERIMENT 11. PROTAMIN. 10 grams of protamin sulphate in 1 litre of solution. 10 milligrams of trypsin and 2.1 cc. of  $\frac{N}{10}$  NaOH to 25 cc.

Time in minutes after mixing.	$t-t_1$	E.M.F. in volts.	$C_{OH^-}$	$x$	$k$ (Monomolecular).	$k$ (Bimolecular).
29		0.832	$23.3 \times 10^{-7}$			
38	0	0.831	$22.4 \times 10^{-7}$	0		
63	25	0.823	$16.25 \times 10^{-7}$	$6.15 \times 10^{-7}$	$559 \times 10^{-5}$	$676 \times 10^{-6}$
78	40	0.818	$13.3 \times 10^{-7}$	$9.1 \times 10^{-7}$	$567 \times 10^{-5}$	$765 \times 10^{-6}$
93	55	0.812	$10.5 \times 10^{-7}$	$11.9 \times 10^{-7}$	$601 \times 10^{-5}$	$920 \times 10^{-6}$
109	71	0.808	$8.9 \times 10^{-7*}$	$13.5 \times 10^{-7}$	$565 \times 10^{-5}$	$954 \times 10^{-6}$
124	86	0.804	$7.6 \times 10^{-7}$	$14.8 \times 10^{-7}$	$545 \times 10^{-5}$	$943 \times 10^{-6}$
132	94	0.802	$7.0 \times 10^{-7}$	$15.4 \times 10^{-7}$	$537 \times 10^{-5}$	$1046 \times 10^{-6}$
13		0.836	$27.3 \times 10^{-7}$			
23	0	0.835	$26.3 \times 10^{-7}$	0		
33	10	0.833	$24.3 \times 10^{-7}$	$2.0 \times 10^{-7}$	$334 \times 10^{-5}$	$313 \times 10^{-6}$
51	28	0.829	$20.7 \times 10^{-7}$	$5.6 \times 10^{-7}$	$371 \times 10^{-5}$	$367 \times 10^{-6}$
72	49	0.826	$18.3 \times 10^{-7}$	$8.0 \times 10^{-7}$	$323 \times 10^{-5}$	$339 \times 10^{-6}$
90	67	0.824	$16.9 \times 10^{-7}$	$9.4 \times 10^{-7}$	$286 \times 10^{-5}$	$316 \times 10^{-6}$
111	88	0.819	$13.8 \times 10^{-7}$	$12.5 \times 10^{-7}$	$317 \times 10^{-5}$	$391 \times 10^{-6}$
123	100	0.817	$12.8 \times 10^{-7}$	$13.5 \times 10^{-7}$	$314 \times 10^{-5}$	$401 \times 10^{-6}$
152	129	0.814	$11.3 \times 10^{-7*}$	$15.0 \times 10^{-7}$	$285 \times 10^{-5}$	$391 \times 10^{-6}$
170	147	0.812	$10.5 \times 10^{-7}$	$15.8 \times 10^{-7}$	$272 \times 10^{-5}$	$389 \times 10^{-6}$
199	176	0.810	$9.7 \times 10^{-7}$	$16.6 \times 10^{-7}$	$246 \times 10^{-5}$	$369 \times 10^{-6}$
232	209	0.805	$7.9 \times 10^{-7}$	$18.4 \times 10^{-7}$	$250 \times 10^{-5}$	$423 \times 10^{-6}$
251	228	0.804	$7.6 \times 10^{-7}$	$18.7 \times 10^{-7}$	$236 \times 10^{-5}$	$411 \times 10^{-6}$

was again taken as indicating the initial concentration and both the time and the changes in alkalinity were reckoned afresh from this point. The constants were calculated for these latter observations in the same way as for the observation made before the further addition of alkali, only the values of  $\beta$  and of  $t_1$  used were those determined after the addition of the alkali.

### III. THEORETICAL CONSIDERATION OF THE RESULTS.

It is at once evident that under certain conditions the transformation follows very closely the monomolecular formula,  $k$  calculated for this formula only varying within the extent of

the experimental error, while  $k$  calculated for the bimolecular formula progressively increases. This is true for Experiment 5 (cf. Table V) and at once shows that the alkali does not play the part of an accessory catalysor, for in this experiment the concentration of the alkali changes several hundred per cent while the value of  $k$  for the monomolecular formula remains nearly constant throughout.

On the other hand, under certain conditions, the transformation follows very closely the bimolecular formula,  $k$  calculated for this formula being practically constant, while  $k$  calculated for the monomolecular formula progressively decreases. This is true for Experiments 1, 3, 6, 8 (cf. Tables I, III, VI and VIII). Under certain intermediate conditions the reaction is monomolecular in its initial stages and bimolecular in its latter stages. This is true for Experiments 2, 4, 7 and 9 (cf. Tables II, IV, VII and IX).

It is at once evident that the condition which determines whether the transformation is monomolecular or bimolecular in character is the hydroxyl concentration. In the tables embodying the results for those experiments in which the order of the reaction changes during its progress, the approximate  $\text{OH}^-$  concentration at which the change in the order of the reaction occurs, is indicated by an asterisk. It will be seen that it is always in the neighborhood of  $10^{-6} \text{ OH}^-$ . At hydroxyl concentrations in excess of this, the reaction is monomolecular, at hydroxyl concentrations which are less than this the reaction is bimolecular. For a small range of intermediate alkalinities, the order of the reaction is indeterminate. This interpretation is further confirmed by Experiments 10 and 11. In these the reaction evidently approaches in its initial stages the monomolecular type, but as the  $\text{OH}^-$  concentration approaches the limit  $10^{-6} \text{ OH}^-$  the order of the reaction becomes indeterminate. Were the system left to itself, judging from the results of the other experiments, the reaction would thereafter approach more and more closely the bimolecular type. Upon adding alkali, however, the reaction again becomes of the monomolecular type, and only as the alkali again approaches the limit  $10^{-6} \text{ OH}^-$  does the order of the reaction become indeterminate or bimolecular.

The bimolecular formula indicates a greater decrease in the vel.



ocity of transformation than is indicated by the monomolecular formula. That this greater decrease in velocity is not due to hydrolysis and consequent destruction of the trypsin, is shown by the fact that it occurs at the same  $\text{OH}^-$  concentration independently of the time consumed in reaching that point, and that it occurs from the very beginning in precisely those solutions which contain the least hydroxyl, and in which, therefore, the destruction of the trypsin is slowest. That it is not due to the depressing effect of the products, is shown by the fact that it occurs from the very beginning of the transformation in certain solutions and that, moreover, if, instead of taking the total amount of alkali present at the beginning of the experiment as proportional to the amount of substrate hydrolyzed, we take the amount initially present less the amount still left in the solution after a considerable lapse of time, we obtain no better agreement with the monomolecular formula. That it is not due to a "lagging behind" of the equilibrium between the electrode and solution is shown by the fact that it occurs in just those solutions in which the change in  $\text{OH}^-$  concentration due to hydrolysis is least rapid. That it cannot be due to purely experimental errors in the determination is shown by the fact that the displacement in the estimated value of the constant for the monomolecular formula is always in one direction while the experimental error would be indifferently positive and negative.

The precise degree of alkalinity at which the reaction changes character is evidently independent of the nature of the substrate, since it is unaltered by substituting for the predominantly acid protein casein, the predominantly basic protein, protamine. The phenomenon is one, therefore, which primarily depends upon some relation which subsists between the alkali and the trypsin and not upon properties peculiar to the substrate or its products. While we do not intend to offer this as a final explanation of these phenomena we may point out that they are similar to those which would be obtained were the true catalysor a hydrolyzable compound of trypsin with  $\text{NaOH}$  (or other base). Were this the case it is evident that so long as there were enough  $\text{NaOH}$  present to combine with all the trypsin, the reaction would be monomolecular (provided the hydrolysis of the substrate were itself a monomolecular reaction, a fact which the observers

quoted in the introduction have established), while if the NaOH present were insufficient to combine with all the trypsin then the amount of catalyst present in the system would be proportional to the concentration of sodium hydrate, and the constant  $k$ , in the monomolecular formula would vary directly as the  $\text{OH}^-$  concentration; that is, the transformation would be represented by a bimolecular formula.

An examination of the experimental data shows that in every case an apparent equilibrium is attained, the rate of change of hydroxyl concentration becoming inappreciable; as this is approached the constants calculated from both formulæ rather suddenly fall off. This equilibrium is, however, probably not a true equilibrium between the substrate and its products for the following reason: The position of equilibrium, as judged by the amount of alkali neutralized, varies very much with the initial concentration of the alkali in the system—the same amount of casein or protamine will evidently neutralize very varying amounts of alkali before reaching apparent equilibrium, according to whether the alkali is initially added in great or small amount. This varying alkali binding power of the system is very much greater than could be accounted for by the action of  $\text{OH}^-$  ions in increasing the alkali-binding power of an amphoteric electrolyte; and moreover occurs when casein is used as a substrate, a protein which attains its maximum alkali-binding power in practically neutral solutions,<sup>1</sup> while one, at least, of its products is an even stronger acid than casein itself (paranuclein).<sup>2</sup>

Furthermore, not only is the position of apparent equilibrium determined by the quantity of alkali initially added to the system but it is likewise shifted if the alkali be added during the progress of the reaction.

We are thus left with two alternatives: either the alkali shifts the point of true equilibrium between the protein and its products and hence also shifts the observed equilibrium, or else the observed equilibrium is a "false" equilibrium, depending upon the sum of the relations between the protein, its products, the trypsin, and the alkali. The former alternative cannot be

<sup>1</sup> Since its neutral salts obey Ostwald's dilution law. Cf. T. Brailsford Robertson: *Journal of Physical Chem.*, xi, p. 542, 1907.

<sup>2</sup> Cf. Gustav Mann: "*Chemistry of the Proteids*," p. 395, 1906.

accepted because in that case work would be performed in changing the  $\text{OH}^-$  concentration and the decomposition of the substrate could not, since the  $\text{OH}^-$  concentration varies during hydrolysis, be monomolecular in character at any stage of the reaction or at any (variable) alkalinity. Taylor's investigations,<sup>1</sup> which were carried out at an alkalinity considerably greater ( $\frac{N}{15000}$ ) than  $10^{-6}$ , as well as our own rule out this alternative and we are forced to the conclusion that the station of rest in the system trypsin + protein + alkali corresponds to a complex equilibrium in which the protein, trypsin and alkali are all involved.

The investigations described above show clearly in what the "optimum" alkalinity of a tryptic digest consists. Evidently at concentrations of alkali below  $10^{-6}$  (at the point at which rosolic acid changes from rose to red)<sup>2</sup> the velocity of the reaction falls off more rapidly than it would were the alkalinity higher; hence alkalinities higher than this secure a greater amount of hydrolysis in a given period of time. A lower limit of the "optimum" alkalinity is thus established. At alkalinities greater than this, further increase in  $\text{OH}^-$  concentration should be without effect until its action in accelerating the destruction of trypsin becomes appreciable. Thus an upper limit of the "optimum" alkalinity is also established; this has been estimated by Taylor to be about  $\frac{N}{15000} \text{OH}^-$ .<sup>3</sup>

Some experiments upon the progressive diminution in acidity of a peptic digestion of casein were also carried out, but while they succeeded in demonstrating that such changes do occur, the total change was so small that accurate determinations could not be successfully made, since any experimental error would form a considerable proportion of the total change indicated. Moreover, in this case the curves indicating the approach to equilibrium of the electrode and the changes in the  $\text{H}^+$  concentration of the solution do not cut one another, but tend continually to diverge and it is, therefore, uncertain to what extent the readings are influenced by the incomplete equilibrium of the electrode; for, in any minimal displacement of equilibrium,

<sup>1</sup> A. E. Taylor: *Univ. of Calif. Publ. Pathol.*, i, p. 7, 1904.

<sup>2</sup> Cf. E. Salm: *Zeitschr. f. physikal. Chem.*, lvii, Heft. 4, 1906.

<sup>3</sup> A. E. Taylor: *Loc. cit.*

the changes taking place in the solution instead of rectifying it, as in alkaline digests, would only magnify the displacement.

#### IV. SUMMARY.

(1) The progressive changes in alkalinity of tryptic digests of casein and of protamine have been followed by means of the gas-chain.

(2) It is found that the progressive changes in  $\text{OH}^-$  concentration of the digests can be expressed by a monomolecular formula when the total  $\text{OH}^-$  concentration is greater than  $10^{-8}$  and by a bimolecular formula when the  $\text{OH}^-$  concentration lies between this and the concentration at neutrality. For a short range of intermediate alkalinities the order of the reaction is indeterminate.

(3) It is pointed out that the above facts are inconsistent with the view that the  $\text{OH}^-$  ions in a tryptic digest play the part of an accessory catalysor, and, while we do not advance this as an explanation of the phenomena, we have nevertheless pointed out that they are such as would be observed were the real catalysor in these systems a hydrolyzable compound of trypsin with the NaOH or other base present in the system.

(4) Reasons have been advanced for believing that the apparent condition of equilibrium, as regards changes in  $\text{OH}^-$  concentration, which is sooner or later reached in these systems, does not consist in a true equilibrium between the protein and its products but in a "false" equilibrium depending upon the sum of the relations between the protein, the trypsin, and the alkali.

# IX. RESEARCHES ON PYRIMIDINS: SYNTHESSES OF SOME NITROGEN-ALKYL DERIVATIVES OF CYTOSIN, THYMIN AND URACIL.

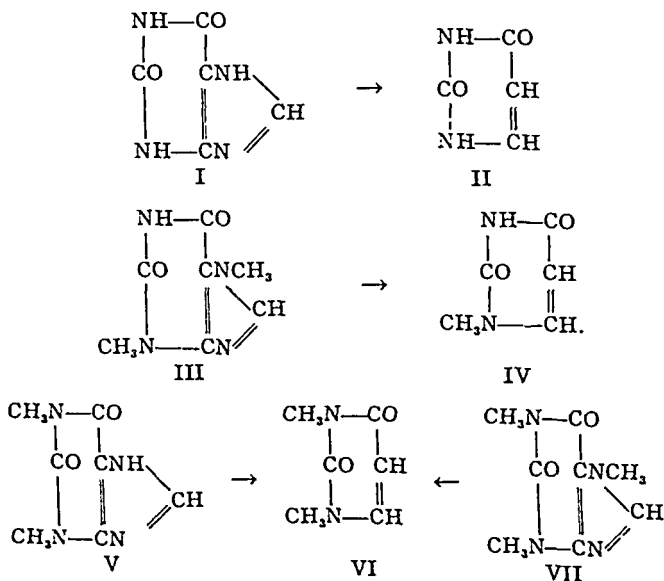
(Thirty-third Paper.)

By TREAT B. JOHNSON AND SAMUEL H. CLAPP.

(From the Sheffield Laboratory of Yale University.)

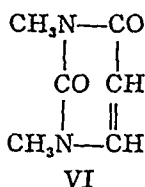
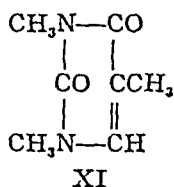
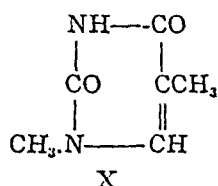
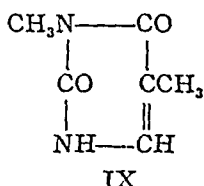
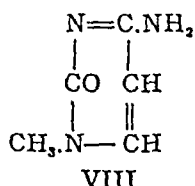
(Received for publication, May 28, 1908.)

The nitrogen-methyl derivatives of cytosin, thymin and uracil are of especial interest because of the occurrence of methylpurins in nature. Purins may be considered as compound rings in which imidazol has been grafted into pyrimidins. A similar relationship exists between 3-methyluracil, IV, 1,3-dimethyluracil, VI, and the purins, theobromin, III, theophyllin, V, and caffen, VII, as exists between uracil, II, and xanthin, I.



These alkyl derivatives are also of interest because of the possibility that future investigations may show the presence of methylpyrimidins in animal or vegetable organisms. It is interesting to note here that Suzuki, Aso and Mitarai,<sup>1</sup> in a paper titled "Ueber die chemische Zusammensetzung der japanischen Sojasauce oder Schoyu," have described a decomposition product of *Schoyu* to which they have assigned the empirical formula  $C_6H_9N_3$ . They state that the compound is probably an isomer of *aminodimethyl pyrimidin*.<sup>2</sup>

We shall describe in this paper the syntheses and properties of 3-methylcytosin, VIII, 1-methylthymine, IX, 3-methylthymine, X, 1,3-dimethylthymine, XI, and 1,3-dimethyluracil, VI.



Two nitrogen-methyl derivatives of thymine and uracil have been described in the literature, viz: 1,3-dimethylthymine, XI, and 1-methyluracil, XII. Dimethylthymine<sup>3</sup> was prepared by heating the mono-potassium salt of thymine with methyl iodide at  $150^\circ$ . We prepared this compound and also 1,3-dimethyluracil, VI, by warming thymine and uracil respectively in alcoholic solution with the required proportions of potassium hydroxide and methyl iodide.

1-Methyluracil, XII, was described in a paper from this laboratory<sup>4</sup> and was prepared in the following manner:

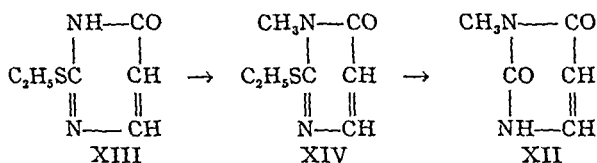
<sup>1</sup> C. Blatt: ii, 1649, 1907. *Bull. College Agr.*, Tokio, vii, 477.

<sup>2</sup> Schwarze: *Journ. f. prakt. Chem.*, xlii, p. 1; Schlenker: *Ber. d. deutsch. chem. Gesellsch.*, xxxiv, p. 2819; Schmidt: *ibid.*, xxxv, p. 1577.

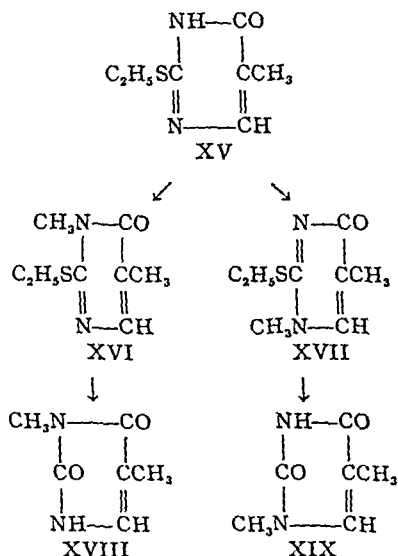
<sup>3</sup> Steudel: *Zeitschr. physiol. Chem.*, xxx, p. 539.

<sup>4</sup> Johnson and Heyl: *Amer. Chem. Journ.*, xxxvii, p. 628.

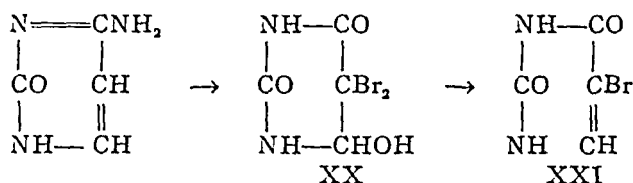
1-methyl-2-pseudoethylthiourea was condensed with the sodium salt of ethyl formylacetate giving 1-methyl-2-ethylmercapto-6-oxypyrimidin, XIV. This same mercaptopyrimidin was also obtained by treatment of 2-ethylmercapto-6-oxypyrimidin, XIII, with methyl iodide in presence of alkali. Hydrolysis of this mercaptopyrimidin with hydrochloric acid gave 1-methyluracil, XII.



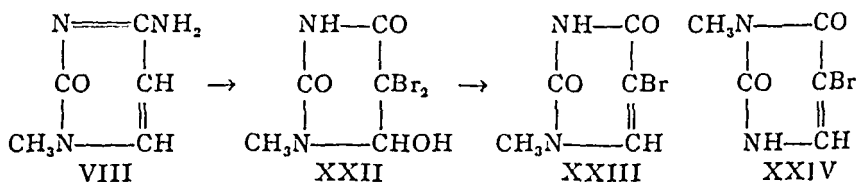
We now find that 2-ethylmercapto-5-methyl-6-oxypyrimidin XV, reacts with methyl iodide in presence of potassium hydroxide giving about equal proportions of the two isomeric pyrimidins—1,5-dimethyl-2-ethylmercapto-6-oxypyrimidin, XVI (m. 65°) and 3,5-dimethyl-2-ethylmercapto-6-oxypyrimidin, XVII (m. 156°). Hydrolysis of these mercaptopyrimidins with concentrated hydrochloric acid gave 1-methylthymine, XVIII (m. 202° to 205°) and 3-methylthymine, XIX (m. 280° to 282°), respectively.



The structures of the isomeric methylthymins and incidentally the corresponding mercaptopyrimidins were established in the following manner: Wheeler and Johnson<sup>1</sup> have shown that cytosin reacts smoothly with bromine water giving oxydibromhydrouracil, XX. When this hydropyrimidin was digested with alcohol it was converted quantitatively into 5-bromuracil, XXI. Since the 6-amino radical is removed by this treatment,



it seemed probable to the writers that substituted cytosin derivatives would behave in a similar manner giving substituted uracils. Our experimental data confirms this assumption. We find that cytosin reacts with methyl iodide in presence of potassium hydroxide giving 3-methylcytosin, VIII. This pyrimidin reacted with bromine water giving a quantitative yield of 3-methyloxydibromhydrouracil, XXII. When this hydropyrimidin was warmed with alcohol it was converted into 3-methyl-5-bromuracil, XXIII, melting at 255° to 260°. The isomeric 1-methyl-5-bromuracil<sup>2</sup> XXIV, melts at 228° to 229°. 5-Methylcytosin apparently reacts with methyl iodide in a similar manner as cytosin giving 3,5-dimethylcytosin, XXV. When this

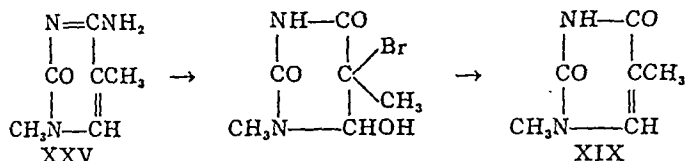


pyrimidin was treated successively with bromine water and alcohol, it was converted quantitatively into 3-methylthymine, XIX, melting at 280° to 282°. This result also proves that the

<sup>1</sup> This *Journal*, iii, p. 183.

<sup>2</sup> Johnson and Heyl: *Loc. cit.*





mercaptopyrimidin, XVII, which melts at  $156^\circ$ , is a 3-methyl derivative since it gives 3-methylthymin on hydrolysis.

The introduction of methyl groups into uracil, thymin, and cytosin, has a similar influence on their physical properties as in the case of purins. They increase the solubility and lower the melting points. For example: while uracil and thymin are difficultly soluble in alcohol, the methyl derivatives of these pyrimidins dissolve easily in this solvent and are moderately soluble in cold water. The 3-methyl derivatives of 2-ethylmercapto-5-methyl-6-oxypyrimidin, thymin and 5-bromuracil are more soluble in water than the isomeric 1-methylpyrimidins.

It is also interesting to note that the 3-methylpyrimidins melted higher, in every series examined, than the isomeric 1-methylpyrimidins:

2-Ethylmercapto-1,5-dimethyl-6-oxypyrimidin.

( $65^\circ$ )

1-Methylthymin.

( $202^\circ$  to  $205^\circ$ )

1-Methyloxynitrohydrothymin.

( $135^\circ$  to  $136^\circ$ )

1-Methyl-5-bromuracil.

( $228^\circ$  to  $229^\circ$ )

2-Ethylmercapto-3,5-dimethyl-6-oxypyrimidin.

( $156^\circ$ )

3-Methylthymin.

( $280^\circ$  to  $282^\circ$ )

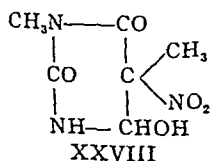
3-Methyloxynitrohydrothymin.

( $178^\circ$  to  $181^\circ$ )

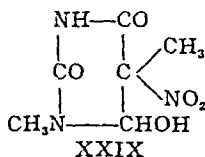
3-Methyl-5-bromuracil.

( $255^\circ$  to  $260^\circ$ )

1-Methylthymin and 3-methylthymin reacted in a similar manner with fuming nitric acid as thymin giving characteristic oxynitrohydrothymins,<sup>1</sup> XXVIII and XXIX.

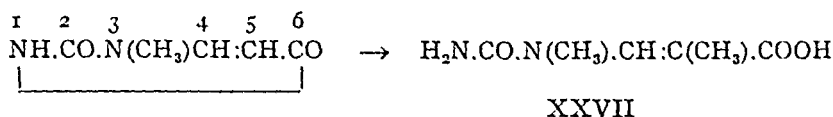
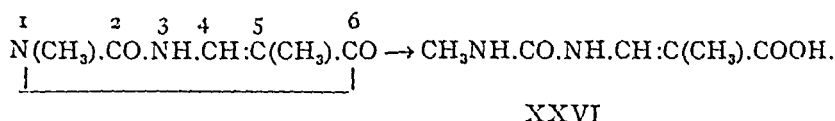


and



<sup>1</sup> Johnson: *Amer. Chem. Journ.*, xl; *This Journal*, iv, p. 407.

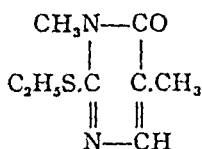
Conductivity measurements on thymine and its methyl derivatives disclosed the interesting facts that thymine and 1,3-dimethylthymine gave practically constant conductivities at 25°. On the other hand, 1-methylthymine and 3-methylthymine gave abnormal conductivities which increased with the length of time these pyrimidines were kept in solution (see Appendix). This interesting behavior is possibly due to a slow hydrolysis of the pyrimidine ring giving, in solution,  $\beta$ -uraminoacrylic acids, XXVI and XXVII.



The methyl derivatives of thymine, described in this paper, should be of interest to the pharmacologist. It is a well known fact that the methylated dioxypurins possess a pronounced diuretic action. Sweet and Levene<sup>1</sup> have recently shown that the administration of thymine to a dog also caused a most pronounced diuresis. Whether the methylated thymine will possess a higher diuretic action than thymine must be decided by further study.

#### EXPERIMENTAL PART.

##### *2-Ethylmercapto-1,5-dimethyl-6-oxypyrimidin:*



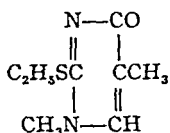
Five grams of 2-ethylmercapto-5-methyl-6-oxypyrimidine and a molecular proportion of potassium hydroxide (1.6 grams) were dissolved in boiling 95 per cent alcohol. An excess of

<sup>1</sup> *Journ. of Exper. Med.*, ix, p. 229.

methyl iodide was then added and the solution boiled for about one hour when it no longer reacted alkaline to turmeric. The undissolved potassium iodide was filtered off and the filtrate heated on the steam-bath to remove the excess of alcohol. We obtained an oily residue, which deposited a mixture of 2-ethylmercapto-1,5-dimethyl-6-oxypyrimidin and unaltered 2-ethylmercapto-5-methyl-6-oxypyrimidin, when triturated with cold water. The filtrate contained the isomeric 2-ethylmercapto-3,5-dimethyl-6-oxypyrimidin (see below). 2-Ethylmercapto-1,5-dimethyl-6-oxypyrimidin was freed from the unaltered material by treatment with a small volume of a cold, dilute solution of sodium hydroxide. The weight of the crude pyrimidin was 2.1 grams or 30 per cent of the theoretical. It was purified for analysis by crystallization from hot water, and separated, on slow cooling, in long, slender prisms which melted at 65° to a clear oil without effervescence. It did not contain water of crystallization. It was dried for analysis over sulphuric acid (Kjeldahl):

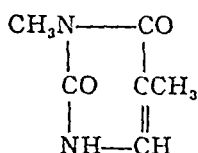
	Calculated for $C_8H_{12}ON_2S$ :	Found:
N.....	15.22	15.17

*2-Ethylmercapto-3,5-dimethyl-6-oxypyrimidin:*



In order to isolate this pyrimidin from the above filtrate, the solution was evaporated to dryness and the residue extracted several times with cold chloroform. When the chloroform was evaporated, at ordinary temperature, 1.8 gram of the crude pyrimidin were obtained, or 33.4 per cent of the theoretical. The compound crystallized from benzene in prisms which melted at 156° to a clear oil without effervescence. It was dried for analysis over sulphuric acid (Kjeldahl):

	Calculated for $C_8H_{12}ON_2S$ :	Found:
N.....	15.22	15.20

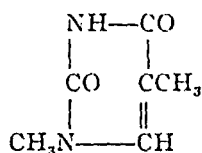
*1,5-Dimethyl-2,6-dioxypyrimidin (1-methylthymine):*

A quantitative yield of this pyrimidin was obtained when 2-ethylmercapto-1,5-dimethyl-6-oxypyrimidin was digested with hydrobromic acid until the evolution of ethylmercaptan ceased (8 hours). The acid solution was then evaporated to dryness and the pyrimidin crystallized from water. It separated in aggregates of stout prisms which melted at  $202^\circ$  to  $205^\circ$ , with effervescence, to a clear oil. The compound was readily soluble in boiling alcohol and acetone. It did not contain water of crystallization. Analysis:

0.2680 gram of substance gave 0.5081 gram of  $\text{CO}_2$  and 0.1379 gram  $\text{H}_2\text{O}$ .

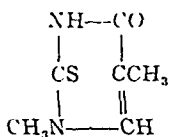
Nitrogen determination (Kjeldahl):

	Calculated for $\text{C}_6\text{H}_8\text{O}_2\text{N}_2$ :	I.	Found: II.	III.
C.....	51.43	51.71		
H.....	5.71	5.71		
N.....	20.00		20.07	20.07

*3,5-Dimethyl-2,6-dioxypyrimidin (3-methylthymine):*

This pyrimidin was not the only product formed when 2-ethylmercapto-3,5-dimethyl-6-oxypyrimidin was digested with hydrobromic acid for 5 hours. When the acid solution was evaporated to dryness, we obtained a mixture of 3-methylthymine and a compound which was difficultly soluble in cold water. The latter was soluble in warm alcohol and crystallized from hot water in needles melting at  $229^\circ$  to  $230^\circ$ , without effervescence to a clear oil. It was soluble in alkalis and gave a strong test for sulphur. The analytical determinations indi-

cated that the compound was *2-thio-3,5-dimethyl-6-oxyypyrimidin*. Analysis (Kjeldahl):

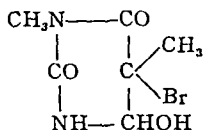


	Calculated for $\text{C}_6\text{H}_8\text{ON}_2\text{S}$ :	Found:	
		I.	II.
N.....	17.95	18.4	18.3

3-Methylthymin is more soluble in cold water than this 2-thiopyrimidin and separated, nearly pure, when the aqueous filtrates (above) were concentrated and cooled. It was purified for analysis by recrystallization from water and melted, when heated slowly, at  $280^\circ$  to  $282^\circ$  to a clear oil. This pyrimidin showed a very characteristic behavior when crystallized from hot water. When the hot, saturated, aqueous solution was quickly cooled, the pyrimidin separated immediately as a bulky, homogeneous mass of long, prismatic needles. On standing, these prisms soon disintegrated, apparently redissolved, and were replaced by characteristic octahedral prisms. The transformation was complete in a few minutes. The compound did not contain water of crystallization. Analysis (Kjeldahl):

	Calculated for $\text{C}_6\text{H}_8\text{O}_2\text{N}_2$ :	Found:
N.....	20.00	19.91

*1-Methyl-5-brom-4-oxyhydrothymin:*

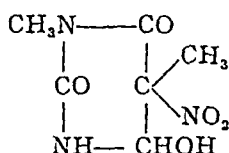


This compound was obtained when 1-methylthymin was dissolved in an excess of bromine water and the solution allowed to evaporate in a vacuum over sulphuric acid. It crystallized from bromine water in stout prisms and melted, on slow heating,

at about  $123^{\circ}$  to  $125^{\circ}$  to a clear oil. It was dried for analysis over sulphuric acid (Kjeldahl):

	Calculated for $C_6H_9O_3N_2Br$ :	Found:
N.....	11.81	11.92

*1-Methyl-5-nitro-4-oxyhydrothymine:*

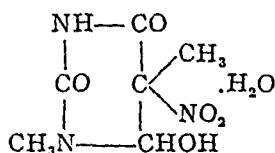


This compound was prepared by dissolving 1-methylthymine in a small volume of fuming nitric acid (sp. gr. 1.5) and allowing the solution to evaporate spontaneously in the air. It deposited in well-developed prisms melting at about  $135^{\circ}$  to  $136^{\circ}$  with effervescence. Analysis:

- I. 0.0844 gram substance gave 15.5 cc. moist  $N_2$  at  $21^{\circ}$  and 728 mm.  
 II. Nitrogen determination (Kjeldahl):

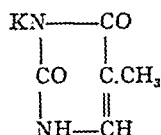
	Calculated for $C_6H_9O_3N_2$ :	Found:	
		I.	II.
N.....	20.69	20.84	21.1

*3-Methyl-5-nitro-4-oxyhydrothymine:*



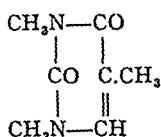
This pyrimidin was prepared in the same manner as its isomer. It separated in large prisms which decomposed at about  $178^{\circ}$  to  $181^{\circ}$  with effervescence. This decomposition point varies according to the rate of heating. A nitrogen determination indicated that the compound contained one molecule of water of crystallization. It slowly underwent decomposition when heated at  $100^{\circ}$ . Analysis (Kjeldahl):

	Calculated for $C_6H_9O_3N_2 \cdot H_2O$ :	Calculated for $C_6H_9O_3N_2$ :	Found:
N.....	19.00	20.69	18.74

*Mono-potassium salt of thymin:*

Finely pulverized thymin and a molecular proportion of potassium hydroxide were dissolved in boiling absolute alcohol and the solution boiled for four hours. The potassium salt separated, on cooling, in long needles. It was purified for analysis by recrystallization from 95 per cent alcohol. Nitrogen determinations in the salt, dried to a constant weight at 110°, agreed with the calculated value in a mono-potassium salt of the closed ring (Kjeldahl):

	Calculated for $\text{C}_5\text{H}_5\text{O}_2\text{N}_2\text{K}$ :	Found:
		I.      II.
N.....	17.07	17.22   17.32

*1, 3-Dimethylthymin:*

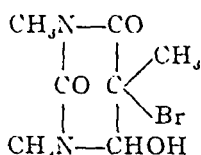
This pyrimidin was first described by Steudel.<sup>1</sup> We obtained the same derivative, in a smooth manner, under the following conditions: Five grams of thymin and 4.6 grams of potassium hydroxide were dissolved in 90 cc. of 95 per cent alcohol and an excess of methyl iodide added to the warm solution. The solution was boiled for twenty minutes, then evaporated to dryness, and the crystalline residue extracted several times with cold chloroform. When the chloroform was evaporated we obtained 2.7 grams of the dimethyl pyrimidin. It was very soluble in water and chloroform and difficultly soluble in ether and petroleum ether. It crystallized from alcohol in

<sup>1</sup> *Loc. cit.*

long needles melting sharply at  $153^{\circ}$  to a clear oil. Analysis (Kjeldahl):

	Calculated for $C_7H_{10}O_2N_2$ :	Found:
N .....	18.18	18.32

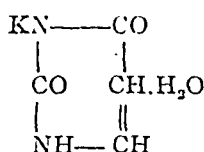
*1,3-Dimethyl-5-brom-4-oxyhydrothymin:*



Was prepared by dissolving 1, 3-dimethylthymin in bromine water. When the aqueous solution was concentrated in a vacuum, the pyrimidin separated in prisms melting at  $132^{\circ}$  to  $133^{\circ}$  to a clear oil. Analysis (Kjeldahl):

	Calculated for $C_7H_{11}O_2N_2\text{Br}$ :	Found:
N .....	11.16	11.25

*Mono-potassium salt of uracil:*



One molecular proportion of potassium hydroxide was dissolved in 450 cc. of absolute alcohol and 13 grams of finely pulverized uracil suspended in the solution. After digesting for eight hours the uracil was completely changed into the potassium salt. It was difficultly soluble in absolute alcohol and very soluble in cold water. It separated from dilute alcohol in balls of long needles. The yield was quantitative. The salt contained one molecule of water of crystallization which was determined by heating at  $120^{\circ}$ .

- I. 0.3365 gram substance lost 0.0351 gram  $\text{H}_2\text{O}$ .  
 II. 0.2871 " " " 0.0302 " "

	Calculated for $C_4H_3O_2N_2K \cdot \text{H}_2\text{O}$ :	Found:
$\text{H}_2\text{O}$ .....	10.70	I. 10.43 II. 10.52



Nitrogen determination in salt dried at 160° (Kjeldahl):

	Calculated for $C_4H_3O_2N_2K$ :	Found:	
		I.	II.
N.....	18.67	18.51	18.55

Potassium determination in the hydrous salt:

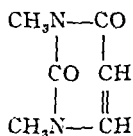
0.1727 gram substance gave 0.0775 gram KCl.

	Calculated for $C_4H_3O_2N_2K.H_2O$ :	Calculated for $C_4H_3O_2N_2K$ :	Found:
K.....	23.21	25.98	23.55

Nitrogen determination in hydrous salt (Kjeldahl):

	Calculated for $C_4H_3O_2N_2K.H_2O$ :	Calculated for $C_4H_3O_2N_2K$ :	Found:
N.....	16.67	18.65	16.81

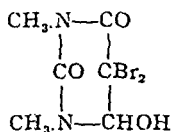
### 1,3-Dimethyluracil:



This compound was prepared by warming, in alcoholic solution, molecular proportions of potassium hydroxide and the potassium salt of uracil with an excess of methyl iodide. After boiling for three hours the solution was evaporated to dryness and the pyrimidin extracted with chloroform. It crystallized from a mixture of alcohol and ether in long, slender prisms which melted at 121° to 122°. It was extremely soluble in cold water, alcohol and chloroform, but insoluble in ether and petroleum ether. Nitrogen (Kjeldahl):

	Calculated for $C_6H_5O_2N_2$ :	Found:		
		I.	II.	III.
N.....	20.00	20.05	20.06	20.24

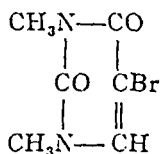
### 1,3-Dimethyl-dibromoxyhydrouracil:



This pyrimidin crystallized from bromine water in microscopic prisms with curved outline. It melted at  $135^{\circ}$  to  $136^{\circ}$  to a clear oil. Analysis (Kjeldahl):

	Calculated for $C_6H_5O_3N_2Br_2$ :	Found:
N.....	8.86	9.0

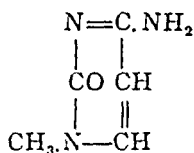
*1, 3-Dimethyl-5-bromuracil:*



This compound was prepared by digesting the above hydro-uracil derivative with absolute alcohol. It was purified for analysis by recrystallization from water and melted at  $181^{\circ}$  to  $182^{\circ}$  to a clear oil. Analysis (Kjeldahl):

	Calculated for $C_6H_7O_3N_2Br$ :	Found:
N.....	12.79	13.0

*2-Oxy-3-methyl-6-aminopyrimidin (3-methylcytosin):*



Six and eight-tenths grams of anhydrous cytosin and 3.4 grams of potassium hydroxide were dissolved in 60 cc. of boiling, absolute alcohol, and 16 grams of methyl iodide added to the solution. After boiling for two hours the solution was evaporated to dryness and the residue of pyrimidin and potassium iodide dissolved in cold water. The iodine was removed with silver sulphate and the excess of silver by precipitation with hydrogen sulphide. The sulphuric acid was then quantitatively removed with barium hydroxide and the filtrate from barium sulphate concentrated to a small volume. The base was precipitated from this solution with a hot, saturated solution of mercuric chloride and the mercury precipitate decomposed in

the usual way with hydrogen sulphide; the chlorine removed with silver sulphate, the excess of silver with hydrogen sulphide, and the sulphuric acid with barium hydroxide. When this solution was evaporated to dryness we obtained about 1.1 gram of the pyrimidin associated with a small amount of unaltered cytosin. The small yield is partly explained by the fact that the base volatilizes with aqueous vapors. It was purified by recrystallization from methylalcohol. It separated in beautiful, distinct prisms which decomposed at about  $278^{\circ}$  to  $279^{\circ}$  to a dark oil. This decomposition point varies according to the rate of heating. The base was extremely soluble in water and did not contain water of crystallization. Analysis (Kjeldahl):

	Calculated for $C_5H_7ON_3$ :	Found:	
		I.	II.
N.....	33.60	33.57	33.9

*The chloroplatinate of 3-methylcytosin:*

Was prepared by adding a solution of platinum chloride to a hydrochloric acid solution of the base. It crystallized from water in long, slender prisms. The salt contained two molecules of water of crystallization which was determined by heating at  $120^{\circ}$ .

I. 0.0513 gram salt lost	0.0028 gram $H_2O$ .
II. 0.2103 " " "	0.0111 " "
III. 0.0841 " " "	0.0045 " "

	Calculated for $(C_5H_7ON_3.HCl)_2.PtCl_4.2H_2O$ :	Found:		
		I.	II.	III.
$H_2O$ .....	5.18	5.45	5.28	5.35

*Platinum determination in anhydrous salt:*

I. 0.0478 gram salt gave	0.0142 gram Pt.
II. 0.1991 " " "	0.0591 " "
III. 0.0793 " " "	0.232 " "

	Calculated for $(C_5H_7ON_3.HCl)_2.PtCl_4$ :	Found:		
		I.	II.	III.
Pt.....	29.54	29.71	29.68	29.26

*Picrate of 3-methylcytosin:*

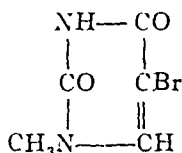
This salt was very insoluble in cold water, and crystallized from hot water in long prisms. The decomposition point varies

greatly according to the rate of heating. When heated slowly it decomposed at about  $280^{\circ}$  with effervescence. Analysis:

0.1351 gram substance gave 28.7 cc.  $N_2$  at  $25^{\circ}$  and 770 mm.

	Calculated for $C_5H_7ON_3 \cdot C_6H_5O_7N_3$	Found:
N.....	23.73	24.0

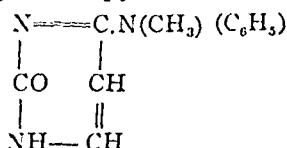
*3-Methyl-5-bromuracil:*



When 3-methylcytosin was dissolved in a little cold water and liquid bromine added to the solution, a heavy precipitate was obtained. This dissolved immediately, on warming, and after heating about ten minutes on the steambath, the solution was evaporated to dryness under diminished pressure. The residue obtained was then dissolved in a small amount of absolute alcohol and the solution boiled for five hours. On cooling, the brompyrimidin separated in needles. It crystallized from hot water in long, slender, distorted needles. They decomposed at about  $255^{\circ}$  to  $260^{\circ}$  to a clear oil with practically no effervescence. The compound was soluble in cold, dilute sodium hydroxide solution and was precipitated again by acetic acid. It gave a strong test for bromine. A mixture of the pyrimidin and the isomeric 1-methyl-5-bromuracil<sup>1</sup> melted at  $175^{\circ}$  to  $195^{\circ}$ . A mixture of the pyrimidin and 5-bromuracil melted from  $230^{\circ}$  to  $270^{\circ}$ . It did not contain water of crystallization. Analysis (Kjeldahl):

	Calculated for $C_5H_5O_2N_2Br$ :	Found:
		I.      II.
N.....	13.66	13.53    13.68

*2-Oxy-6-methylphenylaminopyrimidin:*

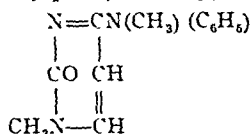


<sup>1</sup> Johnson and Heyl: *Loc. cit.*

Ten grams of 2-ethylmercapto-6-chlorpyrimidin and two molecular proportions of monomethylaniline (twelve grams) were dissolved in dry benzene and the solution boiled for eight hours. The benzene was then evaporated, the residue dissolved in ammonia and the excess of monomethylaniline removed by distillation with steam. When this solution was concentrated we obtained the mercaptopyrimidin as an oil which did not solidify on standing. The crude mercapto derivative was converted into the oxygen derivative by digesting with hydrobromic acid. The yield was 80 per cent of the theoretical. The compound is very insoluble in water and chloroform and crystallizes from alcohol in beautiful hexagonal tables which do not decompose below 285°. Analysis (Kjeldahl):

	Calculated for $C_{11}H_{11}ON_3$ :	Found:
N.....	20.90	20.71

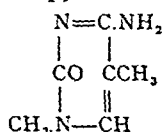
*2-Oxy-3-methyl-6-methylphenylaminopyrimidin:*



Seven and five-tenths grams of 2-oxy-6-methylphenylaminopyrimidin, 2.1 grams of potassium hydroxide, and 12 grams of methyl iodide were dissolved in 150 cc. of absolute alcohol and the solution boiled for two hours. The solution was then evaporated to dryness and the residue extracted with chloroform. When the chloroform was evaporated we obtained the hydriodide of the pyrimidin base. The base was obtained by decomposing the salt with sodium hydroxide and weighed 5.5 grams or 69 per cent of theoretical. It crystallized from water in long, striated prisms which melted sharply at 186° to 187° to a clear oil without effervescence. Analysis (Kjeldahl):

	Calculated for $C_{12}H_{13}ON_3$ :	Found:
N.....	19.53	19.78

*2-Oxy-3,5-dimethyl-6-aminopyrimidin (3,5-Dimethyl-cytosin):*

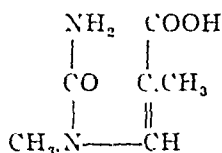


5-Methylcytosin<sup>1</sup> was converted into this base by alkylation with potassium hydroxide and methyl iodide in the usual manner, and the new pyrimidin isolated in the same way as 3-methylcytosin. The compound was extremely soluble in water and volatilized with aqueous vapors. It separated from methylalcohol in prisms. It had no definite melting point, but decomposed from 300° to 310°, according to the rate of heating, with effervescence. Analysis (Kjeldahl):

	Calculated for C <sub>6</sub> H <sub>9</sub> ON <sub>3</sub> :	Found;	
		I.	II.
N.....	30.22	30.09	29.76

*Conversion of 3,5-dimethylcytosin into 3-methylthymin:*

Some 3,5-dimethylcytosin was dissolved in strong bromine water, the solution warmed on the steambath for ten minutes, and then evaporated to dryness in a vacuum. We obtained a crystalline deposit which was digested with absolute alcohol for five hours. The alcohol was then removed by evaporation and the residue redissolved in hot water. Needle-like prisms separated, on cooling, which melted at 160° with effervescence to a colorless oil. This oil immediately solidified on cooling and then melted at 280° to 282° to an oil without effervescence. They did not contain bromine and were soluble in alkalies. We did not obtain enough of this material for analysis but it was probably *β-methyluramino-α-methylacrylic acid*:



When the filtrate (above) was concentrated and cooled the characteristic crystals of 3-methylthymin separated. They melted at 280° to 282° to an oil. A mixture of pure 3-methylthymin and this compound melted at the same temperature. Analysis (Kjeldahl):

	Calculated for C <sub>6</sub> H <sub>9</sub> O <sub>2</sub> N <sub>2</sub> :	Found;
N.....	20.00	20.3

<sup>1</sup> Wheeler and Johnson: *Amer. Chem. Journ.*, xxxi, p. 591.

## CONDUCTIVITY MEASUREMENTS ON THYMIN, 1-METHYLTHYMIN, 3-METHYLTHYMIN, 1,3-DIMETHYLTHYMIN AND 4-METHYLURACIL.

BY N. A. MARTIN.

In a series of conductivity measurements on thymin and its nitrogen-methyl derivatives it was found that the conductivities of thymin and 1,3-dimethylthymin remained nearly constant. The very slight increase in conductivity observed was probably due to small amounts of impurities absorbed from the glass and air. The conductivity of water was found to increase at about the same rate.

On the other hand, duplicate determinations on 1-methylthymin and 3-methylthymin did not give agreeing results nor could dissociation constants be calculated. The investigation showed that the conductivity of these two pyrimidins rose with the length of time they were kept in solution. The greatest rise in conductivity was observed in the determinations on 3-methylthymin.

The measurements were carried out by the customary Kohlrausch method in a thermostat at 25°. The water used was redistilled over barium hydroxide, rejecting all that gave a test for ammonia with Nessler's reagent, until it had a specific conductivity of approximately  $2 \times 10^{-6}$ , and the conductivity vessel, pipettes, etc., were thoroughly steamed before using.

The rise in conductivity was determined by preparing 20 cc. of exactly  $\frac{N}{54}$  solution of the substance directly in the vessel and measuring its conductivity, at intervals, until it remained practically constant. The rise in specific conductivity of pure water, due to absorption of air, solution of soda from the walls of the vessel, etc., was also determined and found to be practically zero (0.072 per hour).

In calculating results the amount of hydrolysis could not be determined as the amount of dissociation of the resulting uraminoacrylic acids (?) was unknown. The specific and molecular conductivities were calculated and the latter plotted as ordinates with the time in hours as abscissas. As a reference line the rise in conductivity of water was also plotted, using 64,000 times the specific conductivity to agree with the molecular conductivity of the solutions ( $\lambda_{64} = 64,000 x$ ).

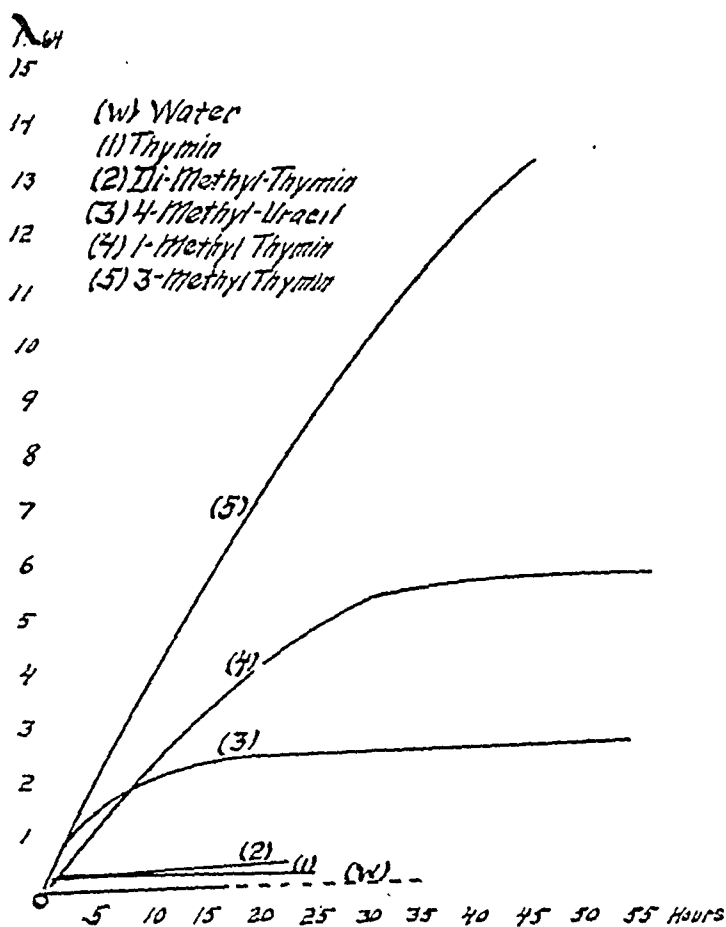
*Molecular conductivity of the pyrimidins in  $\frac{N}{34}$  solution at 25°, showing increase due to hydrolysis.*

Time.	Thymin.	1,3-Dimethyl- thymin.	1-Methylthymin.	3-Methyl- thymin.	4-Methyl- uracil.
$\frac{1}{3}$ hour		0.23			0.35
$\frac{5}{6}$ "					0.75
1 "		0.37	0.49	0.38	
2 "	(+ $\frac{1}{4}$ ) 0.41		(+ $\frac{1}{2}$ ) 1.04	0.90	
3 "	0.42		1.17		
4 "	0.44		1.36		
5 "	0.45		1.54		
6 "	0.46				
7 "	0.47		1.98		
8 "					
17 "		0.68			2.50
18 "		0.69			2.53
19 $\frac{1}{2}$ "		0.72			2.67
20 "		0.74			
22 "		(+ $\frac{1}{2}$ ) 0.75	4.51		
23 "	(+ $\frac{1}{2}$ ) 0.57		4.67		
24 "	0.57		4.96	8.90	
25 "					
26 "					
27 "			5.28	10.19	
28 "			5.56		
30 "			5.75		
41 "				13.53	
49 $\frac{1}{2}$ "					2.97
55 $\frac{1}{2}$ "			6.02		



*Specific conductivity of  $\frac{N}{64}$  pyrimidin solutions at 25°, showing increase due to hydrolysis.*

Time.	H <sub>2</sub> O.	Thymin.	1,3-Di-methyl-thymin.	1-Methyl-thymin.	3-Methyl-thymin.	4-Methyl-uracil.
$\frac{1}{4}$ hour	0.0,21		0.0,36			0.0,55
$\frac{5}{6}$ "						0.0,12
1 "	0.0,22		0.0,57	0.0,77	0.0,60	
2 "	0.0,24	(+ $\frac{1}{2}$ ) 0.0,65		(+ $\frac{1}{2}$ ) 0.0,162	0.0,14	
3 "	0.0,26	0.0,66		0.0,182		
4 "		0.0,67		0.0,212		
5 "		0.0,69		0.0,240		
6 "		0.0,71				
7 "		0.0,72 +		(+ $\frac{1}{2}$ ) 0.0,308		
8 "		0.0,74				
17 "	0.0,55		0.0,107			0.0,39
18 "			0.0,109			0.0,40
19 $\frac{1}{2}$ "						0.0,42
20 "			0.0,113			
22 "			0.0,115	0.0,704		
23 "		(+ $\frac{1}{2}$ ) 0.0,89	0.0,116	0.0,730		
24 "				0.0,775	0.0,139	
25 "						
26 "		0.0,91				
27 "		0.0,92		0.0,824	0.0,159	
28 "		0.0,93		0.0,868		
30 "				0.0,898		
41 "					0.0,211	
49 $\frac{3}{4}$ "						0.0,47
55 $\frac{1}{2}$ "				0.0,991		



# SOME NOTES ON THE EFFICIENCY OF THE FOLIN METHOD FOR THE QUANTITATIVE DETERMINATION OF URINARY AMMONIA.

BY MATTHEW STEEL AND WILLIAM J. GIES.

(From the Laboratory of Biological Chemistry of Columbia University, at the College of Physicians and Surgeons, New York.)

(Received for publication, June 1, 1908).

## I. INTRODUCTION.

Last fall, during the progress of the metabolism research that is described in the paper immediately following this, certain anomalous results were obtained in our quantitative determinations of the urinary ammonia. In the earlier periods of that research ammonia had been determined by the Folin method, in the urines in duplicate for 38 days, with thoroughly concordant results. Shortly after the beginning of a metabolism period, however, during which magnesium sulfate was injected subcutaneously<sup>1</sup> every twenty-four hours, the titrations, in duplicate (at the conclusion of the Folin process as applied to the daily urines), were strikingly discordant, the disagreements amounting to from 1 to 2 cc. of  $\frac{N}{10}$  potassium hydroxid per 25 cc. of urine.

Our inability to obtain satisfactory duplicate results for urinary ammonia content after the magnesium sulfate treatment, or to explain the analytic discrepancies by any probable fault of technique, led us to make two general suppositions regarding the cause of the analytic disagreements observed:

(1) That magnesium was eliminated into the urines in question in relatively large quantities as ammonio-magnesium phosphate, which separated, in part at least, in typically crystalline masses.

<sup>1</sup> Period iv, p. 95 (this volume).

(2) That the *crystalline* ammonio-magnesium phosphate thus deposited was not thoroughly decomposed by sodium carbonate, as used in the Folin process, whereby ammonia, in variable amounts, remained in its solid form as triple phosphate in the urines under investigation.

General examination of the urines that gave the anomalous quantitative results for ammonia content showed at a glance that our first supposition was correct—triple phosphate had crystallized in abundance. In separating portions of the urines for analysis, care had always been taken to isolate fractions of the thoroughly shaken and evenly mixed daily samples. Consequently, we had no reason to believe that any of the above mentioned anomalous results of the ammonia determinations were due to transferal of unequal amounts of the deposited ammonio-magnesium phosphate in the duplicate fractions of the urine taken. We therefore proceeded to test very carefully, and in many trials, the validity of the second supposition stated above.

## II. ANALYTIC OPERATIONS.

*First Series.* Is the amount of sodium carbonate (1 to 2 grams) that is usually taken with 25 cc. of urine in the Folin process sufficient to completely liberate the ammonia from small quantities of crystalline ammonio-magnesium phosphate?

*Method A.* We endeavored to answer this question directly by the following special adaptation of the Folin process: Portions of pure, crystalline ammonio-magnesium phosphate, in different amounts between 50 and 500 milligrams inclusive, were quickly and very accurately weighed on a watch glass and transferred quantitatively to aërometer cylinders of the usual size, through a small dry funnel from which the tube had been removed. All fragments adherent to the watch glass and funnel were brushed into the cylinders. No losses of substance could have occurred in the process. In all the tests the crystalline matter was a comparatively coarse powder. About 25 to 50 cc. of water were poured into the cylinders on the powder, which quickly formed a loose sediment in the undisturbed water. A layer of kerosene was then poured over the liquid in each cylinder merely to duplicate the conditions of the Folin process, although no special frothing could have occurred to require its use. Solid sodium carbonate in definite quantities, ranging between 1 and 4 grams inclusive, was then added to the phosphate-water-kerosene mixtures in the cylinders. The apparatus recommended by Folin was employed for aëration. More

powerful pumps than those recommended by Folin were kept in operation for from five to fifteen hours, so that aëration was unusually effective. In all cases aëration was continued at least five hours. In the groups designated B and C (Table I), aëration was conducted during a second five hour period, or ten hours in all. The aërometer cylinders were not opened between the two periods, but the acid of the first period of absorption was titrated and a new portion of acid substituted for ammonia absorption during the second aëration period. In the sixth determination (Group B) aëration was carried in the same manner through a third period of five hours, or fifteen hours in all. Approximately fifth-normal sulfuric acid was used for the absorption of the ammonia. Congo red was used as the indicator. Our results in the first series of tests are given in Table I.

TABLE I. FIRST SERIES, GROUPS A TO C.

*Pure, crystalline ammonio-magnesium phosphate, 0.05 to 0.5 gram. Sodium carbonate, 1 to 4 grams. Total periods of aëration, 5 to 15 hours. Loss of ammonia: maximum, 48.1 per cent; minimum, 31.1 per cent.*

Group.	Determination.	Weight of $\text{NH}_4\text{MgPO}_4^*$	Weight of $\text{Na}_2\text{CO}_3$	VOLUME OF STANDARD ACID SOLUTION REQUIRED TO NEUTRALIZE—					Ammonia lost.
				AFTER AERATION.				If all $\text{NH}_3$ had been liberated.†	
				5 hrs.	5 hrs.(2)	5 hrs.(3)	Total.		
	no.	gram	grams.	cc.	cc.	cc.	cc.	cc.	per ct.
A.....	1	0.05	1	0.6			0.6	1.05	42.9
	2	0.1	1	1.2			1.2	2.05	41.5
	3	0.2	1	2.4			2.4	4.10	41.5
	4	0.3	1	4.2			4.2	6.15	31.7
B.....	5	0.4	4	4.1	0.55		4.65	8.25	43.6
	6	0.5	4	4.6	1.20	1.30	7.10	10.30	31.1
C.....	7	0.5	1	4.3	1.05		5.35	10.30	48.1
	8	0.5	2.5	5.1	0.80		5.90	10.30	42.7
	9	0.5	4	5.6	0.65		6.25	10.30	39.3

\*  $\text{NH}_4\text{MgPO}_4 \cdot 6\text{H}_2\text{O}$ . A pure crystalline powder obtained from Eimer and Amend. The theoretical content of nitrogen is 5.707 per cent. The average result of seven closely concordant determinations of nitrogen contents by the Kjeldahl method was 5.727 per cent (5.697, 5.766, 5.780, 5.669, 5.725, 5.725, 5.725).

† Seven determinations of the volume of our standard acid solution that was required to neutralize the ammonia liberated in the Kjeldahl process from 0.5 gram samples of the triple phosphate used in this series gave the following results (cc.): 10.25, 10.40, 10.40, 10.20, 10.30, 10.30, 10.30, or an average of 10.30 cc. (See footnote in the summary on page 76).

The first result in Table I shows that one gram of sodium carbonate was unable, after five hours of very strong aëration, completely to eject the ammonia from 50 milligrams of the triple

phosphate. The results for Groups B and C show that, after ten hours' aëration of 0.4 or 0.5 gram samples of triple phosphate with 1 to 4 grams of sodium carbonate, large proportions of ammonium were undisturbed in the crystalline material. Even after fifteen hours' aëration of a 0.5 gram sample of the phosphate with four grams of sodium carbonate, practically one-third of the ammonium remained undisplaced.

These observations gave strong support to our second conclusion regarding the cause of the anomalous ammonia results that prompted this study.

*Second Series.* In the second series of tests, we endeavored to ascertain the effect of previous solution of the triple phosphate on the analytic outcome.

*Method B.* In this process Method A was employed, except that the weighed portion of triple phosphate (0.5 gram) was dissolved in a beaker in a small amount (2 to 4 cc.) of acetic acid (25 per cent) or hydrochloric acid (10 per cent). The acid solution was diluted with about 25 cc. of water and transferred quantitatively to the aërometer cylinder, where it was treated with sufficient sodium carbonate to neutralize it and to afford an excess of 2 to 4 grams of the carbonate. From this point forward Method A was followed to completion. The first two periods of aëration were five hours each or a total of ten hours. In the last four tests of the series (Group E) a third aëration period of fourteen hours was maintained—a total of twenty-four hours of aëration for the individuals of Group E. Results are summarized in Table II.

The data in Table II show clearly that preliminary solution of the triple phosphate did not particularly favor ultimate displacement of the ammonium but that, even in the presence of 4 grams of sodium carbonate and after very strong aëration for twenty-four hours (17), a large proportion of the ammonium remained fixed in the original phosphate.

These observations suggested that the Folin method may be as inadequate for the complete removal of ammonia from small quantities of *dissolved* ammonio-magnesium phosphate as the data of Table I indicated it is for the isolation of ammonia from the *crystalline* form of that substance.

*Third Series.* The outcome of the first two series of tests led us to check the previous results with additional preparations of triple phosphate and also to ascertain the effects of larger proportions of sodium carbonate.

TABLE II. SECOND SERIES, GROUPS D AND E.

Pure, crystalline ammonio-magnesium phosphate (from the supply used in the first series), 0.5 gram. Acid: acetic (25 per cent), 3 to 4 cc. or hydrochloric (10 per cent), 2 cc. Sodium carbonate, 2 to 4 grams. Total periods of aëration, 10 to 24 hours. Loss of ammonia: maximum 52.4 per cent; minimum, 16.5 per cent.

Group.	Determination.	AMMONIO-MAGNESIUM PHOSPHATE.				Weight of $\text{Na}_2\text{CO}_3$ .	VOLUME OF STANDARD ACID SOLUTION REQUIRED TO NEUTRALIZE—						Ammonia lost.
		Weight.	ACID USED TO DISSOLVE IT.		AFTER AÉRATION.				If all $\text{NH}_3$ had been liberated.				
			Kind.	Volume.	5 hrs.		5 hrs. (2)	14 hrs. (3)		Total.			
D.	no.	gram	per cent.	cc.	grams	cc.	cc.	cc.	cc.	cc.	per ct.		
	10	0.5	25 acetic	3	3	5.55	1.00		6.55	10.3*	36.4		
	11	0.5	25 "	4	2	6.2	0.70		6.90	10.3	33.1		
	12	0.5	25 "	4	4	3.6	1.30		4.90	10.3	52.4		
	13	0.5	10 HCl	2	2	6.9	1.05		7.95	10.3	22.8		
	14	0.5	10 "	2	4	5.7	2.00		7.70	10.3	25.2		
E.	15†	0.5	25 acetic	4	4	6.3	0.90	0.40	7.60	10.3	26.2		
	16†	0.5	25 "	4	2	4.4	0.85	0.90	6.15	10.3	40.3		
	17†	0.5	10 HCl	2	4	6.55	1.60	0.45	8.60	10.3	16.5		
	18†	0.5	10 "	2	2	5.60	1.10	0.60	7.30	10.3	29.1		

\*See the second footnote, Table I.

† These determinations were started by the junior author and completed independently by the senior author, who knew nothing of the particular conditions of the tests.

Four preparations were employed for the purpose. The product used in the first two series, purchased from Eimer and Amend, was again selected. A second product was obtained from the Mallinckrodt Chemical Company, a third was a Merck preparation and a fourth was our own make. The Mallinckrodt product was a somewhat finer powder than the others.

We prepared our own product by allowing very dilute ammonium hydroxid to drop slowly from a burette into a crystallization dish containing a fairly concentrated solution of magnesium sulfate and disodium hydrogen phosphate. Deposition occurred gradually and the triple phosphate crystals were well formed and relatively large—much larger than those in the powders obtained from the sources indicated above. The crystalline precipitate was washed with water until it was free from ammonium hydroxid. It was then dried in the open air at room temperature.

The following results were obtained in determinations, by the Kjeldahl method, of the nitrogen contents of our several ammonio-magnesium products:

PRODUCT.	VOLUME OF STANDARD ACID SOLUTION REQUIRED TO NEUTRALIZE THE LIBERATED AMMONIA FROM 0.5 GRAM OF $\text{NH}_4\text{MgPO}_4$								Percentage of nitrogen.	
	1	2	3	4	5	6	7	Avg.	Found	Theory
	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	Avg.	
Eimer and Amend*	10.25	10.40	10.40	10.20	10.30	10.30	10.30	10.30	5.727	5.707
Mallinckrodt . . . . .	13.50	13.50						13.50	7.503	5.707
Merck . . . . .	10.05	10.15						10.10	5.614	5.707
Our own . . . . .	10.00	9.95						10.00	5.522	5.707

\* The actual titration results were: 0.1 gram, 2.05 cc.; 0.2 gram, 4.15 cc.; 0.5 gram (four samples), 10.4, 10.2, 10.3, and 10.3 cc.; 1 gram, 20.6 cc.

With the exception of the Mallinckrodt product, the preparations were quite pure. The nature of the conspicuous impurity in the Mallinckrodt product was not determined. It had no important influence on the tests, however.

In this series of tests, on the four products referred to in the above summary, Method A was employed. Two successive five-hour periods of aëration were conducted in all the tests except one, in which the sample was subjected to uninterrupted aëration for eighteen hours. Results are summarized in Table III.

Table III makes it evident that the results of the first and second series of tests with the Eimer and Amend product were not due to any peculiarities of that particular sample of triple phosphate. In each group of tests the percentage loss of ammonia decreased somewhat with each increase in the amount of sodium carbonate in the aërometer cylinder, but it was always above 20 per cent, even when 10 grams or more of sodium carbonate were used for the decompositions, and aëration was continued ten hours.

*Fourth Series.* At this point it occurred to us that possibly we were overlooking some defect in technique that could be detected by carrying out some of the tests with caustic alkali as the decomposing agent. Furthermore, such a check on our former results promised also to give us additional evidence pertaining to the total ammonia contents of our products. Method A was employed for the tests, but sodium hydroxid was substituted for sodium carbonate. Aëration was continued only three hours. Results are given in Table IV.



TABLE III. THIRD SERIES, GROUPS F TO I.

*Crystalline ammonio-magnesium phosphate (four different preparations), 0.5 gram. Sodium carbonate, 1 to 20 grams. Total period of aëration, 10 hours or 18 hours. Loss of ammonia: maximum, 53.3 per cent; minimum, 22.8 per cent.*

Group.	Determination.	NH <sub>4</sub> MgPO <sub>4</sub> .		Weight of Na <sub>2</sub> CO <sub>3</sub> .	VOLUME OF STANDARD ACID SOLUTION REQUIRED TO NEUTRALIZE—						Ammonia lost.
		Product.	Weight.		AFTER AERATION.				If all NH <sub>3</sub> had been liberated		
					5 hrs.	5 hrs. (2).	Total.	Average.			
	no.		gram.	grms.	cc.	cc.	cc.	cc.	cc.	pr. ct.	
F.....	19	Eimer and Amend	0.5	2	4.50	1.05	5.55				
	20	"	0.5	2	4.20	2.10	6.30				
	21	"	0.5	2	4.80	1.10	5.90				
	22	"	0.5	2	4.90	0.95	5.85	5.90	10.3	42.7	
	23	"	0.5	5	6.10	0.85	6.95				
	24	"	0.5	5	6.90	0.15	7.05	7.00	10.3	32.0	
	25	"	0.5	10	6.50	0.50	7.00				
	26	"	0.5	10	7.05	0.70	7.75				
	27	"	0.5	10	7.75	0.75	8.50	7.75	10.3	24.8	
	28	"	0.5	15	7.10	0.80	7.90				
	29	"	0.5	15	7.20	0.65	7.85	7.87	10.3	23.8	
	30*	"	0.5	15			7.85				
G.....	31*	"	0.5	15			7.95	7.90	10.3	23.3	
	32	Mallinckrodt	0.5	5	5.05	1.20	6.25				
	33	"	0.5	5	5.05	1.30	6.35	6.30	13.5	53.3	
	34	"	0.5	10	7.30	0.80	7.90				
	35	"	0.5	10	6.60	1.50	8.10	8.00	13.5	40.7	
	36	"	0.5	15	7.20	1.20	8.40				
	37	"	0.5	15	8.00	1.20	9.20	8.80	13.5	34.8	
	38	"	0.5	20	9.15	0.60	9.75				
H.....	39	"	0.5	20	8.05	1.10	9.15	9.45	13.5	30.0	
	40	Merck	0.5	1	5.70	0.70	6.40				
	41	"	0.5	1	4.90	1.30	6.20	6.30	10.1	37.6	
	42	"	0.5	2	6.30	0.45	6.75				
	43	"	0.5	2	5.90	0.95	6.85	6.80	10.1	32.7	
	44	"	0.5	5	6.05	0.55	6.60				
	45	"	0.5	5	5.95	1.25	7.20	6.90	10.1	31.7	
	46	"	0.5	10	6.30	0.95	7.25				
I.....	47	"	0.5	10	8.10	0.25	8.35	7.80	10.1	22.8	
	48	Our own	0.5	5	4.20	1.05	5.25				
	49	"	0.5	5	4.35	1.40	5.75				
	50	"	0.5	5	5.60	0.60	6.20				
	51	"	0.5	5	7.05	0.15	7.20	6.10	10.0	39.0	
	52	"	0.5	10	5.05	1.15	6.20				
	53	"	0.5	10	4.90	1.20	6.10				
	54	"	0.5	10	5.60	0.55	6.15				
	55	"	0.5	10	6.05	0.90	6.95	6.35	10.0	36.5	

\* 30 and 31. Two samples of the Eimer and Amend product of 0.5 gram each were aerated for eighteen hours. They required on titration respectively 7.55 cc. and 7.95 cc.; average, 7.90 cc. The ammonia loss was 23.3 per cent.

## 78 The Quantitative Determination of Ammonia

TABLE IV. FOURTH SERIES, GROUP J.

*Crystalline ammonio-magnesium phosphate (four different preparations as in the third series), 0.5 gram. Sodium hydroxid (1 gram) instead of sodium carbonate. Period of aëration, 3 hours. Loss of ammonia: none.*

Group.	Determination.	$\text{NH}_4\text{MgPO}_4$ .		NaOH.	VOLUME OF STANDARD ACID SOLUTION REQUIRED FOR NEUTRALIZATION.		Ammonia lost.
		Product.	Weight.		After aëration 3 hours.	If all $\text{NH}_3$ had been liberated.	
	no.		gram	gram	cc.	cc.	
J...	56	Eimer and Amend	0.5	1	10.25	10.30	none
	57	Mallinckrodt ....	0.5	1	13.50	13.50	"
	58	Merck .....	0.5	1	10.10	10.10	"
	59	Our own .....	0.5	1	9.95	10.00	"

The data in Table IV prove conclusively that the basis for all our calculations in the previous tests was correct, that our technique was not necessarily responsible for the variations in, nor the extent of, the ammonia losses, and that the total ammonia contents of our products were easily discharged completely in a short time by the caustic alkali employed.

These results reinforced the conclusions drawn from the data obtained in the first three series of tests and seemed to show conclusively that sodium carbonate, in the amounts ordinarily used in the Folin process as applied to urine, may fail to eject completely the ammonia from the contained ammonio-magnesium phosphate.

Practically all the previously recorded data were obtained by the junior author under the senior author's guidance. In order to exclude any previously undiscovered error in technique, nearly all the remaining series of tests were conducted either by the senior author unaided, or, as will be indicated here and there, in part by each author; in which special cases the one worker had no knowledge of the conditions of the tests as imposed by the other. In this way it was hoped to exclude entirely every influential error of manipulation or deduction. The results seem to show that this hope was realized.

The checking process was conducted with a *new standard acid*

solution, which was not quite equal in strength to the acid solution used in the first four series. The second series of results for nitrogen content, as determined by the Kjeldahl method, are appended:

PRODUCTS*	VOLUME OF STANDARD ACID SOLUTION REQUIRED TO NEUTRALIZE THE LIBERATED AMMONIA.		
	1.	2.	Average
	cc.	cc.	cc.
Eimer and Amend.....	10.80	10.70	10.75
Mallinckrodt.....	13.85	13.85	13.85
Merek.....	10.30	10.40	10.35
Our own.....	10.20	10.20	10.20

\* Same as those used in the third and fourth series of tests.

*Fifth Series.* Method A was employed in this series. The same amounts of phosphate and carbonate were used in all the tests. The amount of carbonate was relatively larger than in most of the previous groups. Each set of determinations was subjected to a single definite period of aëration (5 to 15 hours). Results are recorded in Table V.

The results summarized in Table V make it very evident that, in harmony with the previous results, large proportions of sodium carbonate (30 of the carbonate to 1 of the phosphate), even after fifteen hours of very vigorous and thorough aëration, failed to effect removal of all the ammonia. It is noticeable that the loss of ammonia after five hours of aëration of 0.5 gram of triple phosphate with 15 grams of sodium carbonate was appreciably greater than that in the tests which included aëration for ten hours.

*Sixth Series.* The influence of different proportions of sodium carbonate on 0.5 gram samples of the same triple phosphate (our own product) is shown unmistakably in the results of our sixth series of tests (Table VI).

Method A was employed for the tests of this series, but, instead of adding solid sodium carbonate to the usual amount of phosphate-water mixture, the weighed quantities of the triple phosphate were transferred to the aërometer cylinders and, after all connections had been closed, 100 cc. of a solution containing the desired amount of sodium carbonate were poured down the inlet tube upon the phosphate, and aëration was imme-

## 80 The Quantitative Determination of Ammonia

diately begun in the absence of the layer of oil (p. 82). This volume of decomposition liquid which was larger than usual, was necessary for the complete retention of sodium carbonate in solution. During aëration of the mixtures containing 20 or 25 grams of sodium carbonate, a special tendency to incrustation of the tip of the inlet tube was manifested after several hours. In a similar series that could not be carried to completion, aëration was entirely stopped in such mixtures by the filling of the tip of the inlet tube with solid sodium carbonate, which formed from the drying of layers of the solution that were deposited in the lower end of

TABLE V. FIFTH SERIES, GROUPS K TO M.

*Crystalline ammonio-magnesium phosphate (from different products, as in the third and fourth series), 0.5 gram. Sodium carbonate, 15 grams. Periods of aëration, 5, 10 or 15 hours. Loss of ammonia: maximum, 32.1 per cent; minimum, 7.8 per cent.*

Group.	Determination.	NH <sub>4</sub> MgPO <sub>4</sub> .		Weight of Na <sub>2</sub> CO <sub>3</sub> .	VOLUME OF STANDARD ACID SOLUTION REQUIRED TO NEUTRALIZE.					Ammonia lost.
		Product.	Weight.		AFTER AERATION.				If all NH <sub>3</sub> had been liberated.	
					5 hrs.	10 hrs.	15 hrs.	Total.		
	no.		gram	grms.	cc.	cc.	cc.	cc.	cc.	pr. ct.
K . . . . .	60	Eimer and Amend	0.5	15			9.5	9.5	10.75	11.6
	61	Mallinckrodt . . . .	0.5	15			12.3	12.3	13.85	11.2
	62	Merck . . . . .	0.5	15			9.2	9.2	10.40	11.5
	63	Our own . . . . .	0.5	15			9.4	9.4	10.20	7.8
L . . . . .	64	Eimer and Amend	0.5	15		9.65		9.65	10.75	10.2
	65	Mallinckrodt . . . .	0.5	15		11.15		11.15	13.85	9.5
	66	Merck . . . . .	0.5	15		9.45		9.45	10.40	9.1
	67	Our own . . . . .	0.5	15		9.00		9.00	10.20	11.8
M . . . . .	68	Eimer and Amend	0.5	15	8.95			8.95	10.75	16.7
	69	Mallinckrodt . . . .	0.5	15	9.40			9.40	13.85	32.1
	70	Merck . . . . .	0.5	15	8.35			8.35	10.40	19.7
	71	Our own . . . . .	0.5	15	8.65			8.65	10.20	15.2

the inlet tube as the solution rose and fell with fluctuations of pressure. In the sixth series such incrustation was prevented from interfering with aëration by an occasional cessation of the process for a few seconds. The alkaline solution then rose in the inlet tube and the carbonate crust was dissolved. Some time elapsed, after aëration had been resumed, before discontinuance for the same reason again became advisable. These facts are mentioned merely to show clearly that in this series we used excessive proportions of sodium carbonate.

TABLE VI. SIXTH SERIES, GROUPS N AND O.

Pure, crystalline ammonio-magnesium phosphate (our own product, p. 75), 0.5 gram. Sodium carbonate, 5 to 25 grams. Periods of aëration, 4 hours or 10 hours (in two periods of 5 hours). Loss of ammonia: maximum, 34.3 per cent; minimum, 3.4 per cent.

Group.	Determination.	Weight of NH <sub>4</sub> MgPO <sub>4</sub> .	of Na <sub>2</sub> CO <sub>3</sub> .	VOLUME OF STANDARD ACID SOLUTION REQUIRED FOR NEUTRALIZATION.					Ammonia lost.
				AFTER AERATION.			Total.	If all NH <sub>3</sub> had been liberated	
				4 hrs.	5 hrs. (1).	5 hrs. (2).			
	no.	gram.	grams.	cc.	cc.	cc.	cc.	cc.	pr. ct.
N.....	72	0.5	20	9.75			9.75	10.2	4.4
	73	0.5	5	6.70			6.70	10.2	34.3
O*.....	74	0.5	10		6.85	0.70	7.55	10.2	26.0
	75	0.5	15		8.40	0.55	8.95	10.2	12.3
	76	0.5	20		9.50	0.10	9.60	10.2	5.9
	77	0.5	25		9.85	0.00	9.85	10.2	3.4

\* The carbonate-phosphate mixtures in this series showed increasing clearness with the rising concentration of carbonate. In test 77 the turbidity was almost negligible, showing that practically all the triple phosphate had gone into solution. In dilute NaOH (Table III) the product dissolved completely.

The results summarized in Table VI make it apparent that even in the presence of 50 times its weight of sodium carbonate, and after 10 hours of thorough aëration, crystalline ammonio-magnesium phosphate cannot be completely decomposed by the Folin method of ammonia determination.

*Seventh Series.* At this point we made additional checks of our manipulations and experimental conditions as is indicated in Table VII. Method A was used—without triple phosphate in Group P; with ammonium chlorid, instead, in Group Q.

The data in Table VII finally removed our suspicion that our results were due to impure chemicals, faulty technique or unsuspected errors.

*Eighth Series.* This investigation was concluded with a determination of the effects of relatively very great excesses of sodium carbonate in the aëration process. The results of this final test, which are given in Table VIII, merely confirmed the conclusion

# 82 The Quantitative Determination of Ammonia

TABLE VII. SEVENTH SERIES, GROUPS P AND Q.

*Tests of the purity of the sodium carbonate, and of the possible disturbing influence of the kerosene, used in the first six series. Checks on the aëration process and on the handling of the Folin method in general.*

Group.	Determination.	Aëration 4 hrs. in the absence of $\text{NH}_4\text{MgPO}_4$ but in the presence of—	STANDARD SOLUTION OF ACID.			Ammonia lost.
			Used.	Recovered.	Neutralized.	
	no.		cc.	cc.	cc.	
P.....	78	Water alone .....	15.0	15.05	none	
	79	Water + 15 grams $\text{Na}_2\text{CO}_3$ ....	15.0	15.00	"	
	80	Water + 15 grams $\text{Na}_2\text{CO}_3$ + kerosene .....	15.0	15.05	"	
Q.....		10 cc. $\frac{N}{5}$ $\text{NH}_4\text{Cl}$ .....				
	81	a. Without kerosene .....	15.0	10.00	5.00	none
	82	b. Without " .....	15.0	10.05	4.95	"
	83	c. With " .....	15.0	10.00	5.00	"
	84	d. With " .....	15.0	10.00	5.00	"

TABLE VIII. GROUP R.

*Pure, crystalline ammonio-magnesium phosphate (Eimer and Amend product), 0.05 gram. Sodium carbonate, 2 to 16 grams. Periods of aëration (2), 5 hours. Loss of ammonia: maximum 35.19 per cent; minimum, 12.04 per cent.*

Group.	Determination.	Weight of NH <sub>4</sub> MgPO <sub>4</sub>	Weight of Na <sub>2</sub> CO <sub>3</sub>	VOLUME OF STANDARD ACID SOLUTION REQUIRED TO NEUTRALIZE—				Ammonia lost.
				AFTER AERATION.			If all NH <sub>3</sub> had been liberated	
				5 hrs.	5 hrs. (2)	Total.		
R.....	no.	gram.	gram.	cc.	cc.	cc.	cc.	per cent.
	85	0.05	2	0.65	0.05	0.70	1.08	35.19
	86	0.05	4	0.60	0.15	0.75	1.08	30.56
	87	0.05	8	0.70	0.10	0.80	1.08	25.93
	88	0.05	16	0.80	0.15	0.95	1.08	12.04

already drawn that Folin's splendid method fails, in the case of triple phosphate, to give perfectly accurate results for ammonia content.

We hope in the near future to report a simple modification of the Folin method that will yield all the ammonia from ammonio-magnesium phosphate in urine without producing ammonia from non-ammoniacal radicals.





# A STUDY OF THE INFLUENCE OF MAGNESIUM SULFATE ON METABOLISM

By MATTHEW STEEL.

(From the Laboratory of Biological Chemistry of Columbia University, at the  
College of Physicians and Surgeons, New York.)

(Received for publication, May 7, 1908.)

I. Introduction.....	85
II. General description of the experiments.....	86
III. First experiment. First <i>metabolism</i> experiment.....	88
IV. Second experiment. Does bone ash in the diet offset the cathartic influence of magnesium sulfate given with the food?	98
V. Third experiment. A determination of the conditions most favorable for intravenous injection of large doses of magne- sium sulfate into dogs.....	101
VI. Fourth experiment. Second <i>metabolism</i> experiment.....	102
VII. Fifth experiment. Does the intramuscular injection of large doses of magnesium sulfate into dogs cause the develop- ment of abscesses?.....	118
VIII. General comparison and discussion of the results.....	118
IX. Summary of general conclusions.....	123

## I. INTRODUCTION.

Magnesium is normally present in the tissues and fluids of all plants and animals. Comparatively little is known, however, regarding the rôle that magnesium plays in the manifestations of life phenomena, particularly in animals. Some of its salts are widely used therapeutically, to produce purgative effects especially.

Many investigators have studied the influence of magnesium salts upon animals, particularly after the injection of such salts by one method or another. The greater number of the *injection* experiments with magnesium salts have been carried out during the past thirty-five years by investigators who have, in effect,

inquired into the truth of the statement by Luton<sup>1</sup> that subcutaneous injections of *small* doses of magnesium sulfate produce purgation. Very discordant conclusions have been drawn meanwhile by the authors of the subsequent papers on this particular phase of the subject. Recke<sup>2</sup> and Hay<sup>3</sup> reported that subcutaneous injections of magnesium sulfate in relatively *large* doses may be fatal to animals. Hay stated that death occurs under such circumstances from respiratory paralysis.

Lately Meltzer<sup>4</sup> and collaborators have shown that magnesium sulfate, if subcutaneously injected in relatively large though non-lethal doses, produces profound anesthesia without excitation, but with complete relaxation of all the voluntary muscles, and with abolition of many reflex acts. The animal ordinarily recovers completely from the anesthesia produced by such non-lethal doses.

These and the many additional observations by Meltzer and others led Dr. Gies to suggest an investigation of the *metabolic* effects of magnesium sulfate. A careful review of the literature indicated that no extensive metabolic study of this kind had ever been attempted before this work was inaugurated.<sup>5</sup>

## II. GENERAL DESCRIPTION OF THE EXPERIMENTS.

This investigation consisted of two long metabolism experiments, in which food and excreta were analyzed: and several collateral experiments, in which no analyses were made. The metabolism experiments were carried out by the methods usually employed in this laboratory.<sup>6</sup>

**ANIMALS AND ENVIRONMENT.** All the experiments were performed on full grown, healthy dogs carefully selected to suit the special conditions

<sup>1</sup> Luton: *Gazette hebdomadaire de médecine et de chirurgie*, xxviii, p. 455, 1874.

<sup>2</sup> Recke: Dissertation, Göttingen, 1881.

<sup>3</sup> Hay: *Journ. of Anat. and Physiol.* vxii, p. 512, 1883.

<sup>4</sup> Meltzer and Auer: *Proc. of the Soc. for Exper. Biol. and Med.*, ii, p. 81, 1905; *Amer. Journ. of Physiol.*, xiv, p. 366, 1905.

<sup>5</sup> Yvon: *Arch. de Physiol.*, xxx, p. 304, 1898; Winterberg: *Jahresber. der Tierchemie*, xxxiii, p. 799, 1903; Malcolm: *Journ. of Physiol.*, p. 183, 1905.

<sup>6</sup> Mead and Gies: *Amer. Journ. of Physiol.*, v, p. 106, 1901; also, Gies and collaborators: *Biochemical Researches*, i, p. 419 (Reprint No. 21), 1903.

of the experiments. In each case we were particularly fortunate in our selections.

The animals under observation were confined in cages devised in this laboratory and recently described.<sup>1</sup>

**Food.** The daily food consisted of a mixture of hashed lean beef, lard, cracker meal, bone ash, and water. The raw beef was taken from large supplies preserved in a frozen condition.<sup>2</sup>

The daily portion of cracker meal was obtained from large quantities of the commercial product kept in big bottles with ground glass stoppers. Lard was purchased in small amounts and kept in a refrigerator. It was always devoid of rancidity. The bone ash consisted of the thoroughly incinerated, carbon free commercial product.<sup>3</sup> Ordinary tap water was used. In the first experiment the dog was given his daily portion of food at 12:15 p.m.; in the second, at 11:30 a.m.

**PERIODS, WEIGHTS.** In our metabolism experiments each day of the first experiment ended at 12:15 p.m.; of the second experiment, at 11:30 a.m. The dogs were weighed just before they were fed. A new period was always begun with the day on which the dog was subjected to new conditions. The periods were made relatively long to afford more reliable "daily average" results of analysis.

**COLLECTION OF EXCRETA.** We collected the urine and feces daily just before feeding. No artificial means such as catheterization were employed in this connection. The cage was washed at the end of each period and the total content of nitrogen in the washings determined. This total was added to the total nitrogen of the urine for the corresponding period.

**ANALYSIS.** The total content of nitrogen in the ingredients of the food was determined. The urine was carefully preserved with thymol. Urinary nitrogen, in the leading forms, was determined as follows: ammonia, each day; total, urea and creatinin, every other day; and allantoin, uric acid and purin bases, at the end of each period.

**ANALYTIC METHODS.** Total nitrogen was determined by the Kjeldahl process, oxidation being effected by means of concentrated sulfuric acid, aided by a little cupric sulfate. Ammonia, urea, creatinin and uric acid were determined by the methods devised by Folin.<sup>4</sup> Allantoin was determined by the Loewi<sup>5</sup> method. Nitrogen of purin bases was determined by a combination of the Arnstein<sup>6</sup> and Salkowski<sup>7</sup> methods.

---

<sup>1</sup> Gies: *Amer. Journ. of Physiol.*, xv, p. 403, 1905.

<sup>2</sup> Gies: *Amer. Journ. of Physiol.*, v, p. 235, 1901; also Gies and collaborators: *Biochemical Researches*, i, p. 69 (Reprint No. 1), 1903.

<sup>3</sup> Steel and Gies: *Amer. Journ. of Physiol.*, xx, p. 343, 1907.

<sup>4</sup> Folin: *Amer. Journ. of Physiol.*, xiii, p. 45, 1905.

<sup>5</sup> Loewi: *Arch. f. exp. Path. u. Pharm.*, xlv, p. i, 1900.

<sup>6</sup> Arnstein: *Zeit. f. physiol. Chem.*, xxiii, p. 417, 1897.

<sup>7</sup> Salkowski: *Salkowski's Manual of Physiol. Chem. and Path.*, Transl. by Orndorff, p. 24, 1904; *Arch. f. d. ges. Physiol.*, lxi, p. 268, 1897-98.

## 88 Influence of Magnesium Sulfate on Metabolism

Magnesium in bone ash and feces was determined as follows: 2.5 grams of the fine powder that had been dried to constant weight, were dissolved in 50 cc. of HCl (sp. gr. 1.15), evaporated to dryness and desiccated at 110° C. The dry residue was dissolved in a few cc. of HCl and about 50cc. of water. The solution was filtered, the filtrate made up to 250 cc. and 50 cc. portions of this solution taken for analysis. A quantity of ferric chlorid sufficient to precipitate the phosphoric acid was added to the solution, after which the mixture was made slightly alkaline with ammonium hydroxid. About two grams of sodium acetate were next added, the solution then boiled a few moments and filtered. The filtrate was treated with a moderate excess of ammonium oxalate to precipitate the calcium, heated, filtered hot, the filtrate evaporated to about 50 cc. and neutralized with HCl. A moderate excess of disodium hydrogen phosphate was added; ten minutes later 10 cc. of strong ammonium hydroxid were added, and the mixture allowed to stand overnight. The product was incinerated and weighed as  $Mg_2P_2O_7$ .

MAGNESIUM SULFATE. The magnesium sulfate employed in all the experiments was a sample of a Kahlbaum ("K") product.

### III. FIRST EXPERIMENT. FIRST METABOLISM EXPERIMENT.

The dog selected for this experiment was a healthy male, weighing about 10 kilos.

DIET. The character of the diet was practically the same throughout the whole experiment. Three samples of meat were used, but all had essentially the same nitrogen content. The amount and composition of the daily diet is indicated in Table I.

TABLE I.  
*Composition of the daily diet.*

INGREDIENTS.	HASHED LEAN BEEF.			CRACKER MEAL.	LARD.*	BONE ASH.	WATER.
Days.....	1-7	8-10	11-12		1-12		
	grams.	grams.	grams.	grams.	grams.	grams.	cc.
Daily amounts.....	150	150	150	40	30	20	350
Nitrogen.....	5.31	5.91	5.34	0.650	0.008	0.006	

\*Only one sample of lard was analyzed. The figure obtained for content of nitrogen was practically the same as that heretofore noted for the same brand of lard that has long been in use in this laboratory.

PREPARATORY PERIOD. Eleven days on the above diet sufficed to accustom the dog to the food and environment, and afforded ample opportunity to observe the general characteristics of the animal. During this time

the weight of the dog rose from 9.80 kilos to 10.16 kilos. The preparatory period ended on November 10, 1907, and on that day, at 12:15 p.m., the accumulation of analytic data was begun. The main part of the experiment was continued uninterruptedly thereafter until noon of December 27, 1907 (47th day). A general supplementary period was maintained until January 11, 1908 (62d day).

The experiment was divided into six periods of different lengths and conditions as follows:

FIRST PERIOD. *Normal conditions. Days 1-10; November 11-20, 1907.* During this period the animal was entirely accustomed to his environment and normal conditions were maintained uniformly.

SECOND PERIOD. *Influence of magnesium sulfate administered per os. Days 11-21; November 21-December 1.* On the 11th day the first dose of magnesium sulfate was given *per os* just before feeding. Dosage in this instance, as in all subsequent administrations by mouth, was effected by enclosing the magnesium sulfate in a little ball of the weighed hashed meat, which the animal swallowed quickly without masticating; and consequently, also without tasting the magnesium compound. The remainder of the food was offered immediately afterward and eaten at once.

We desired to ascertain the effects of magnesium sulfate on the partition of nitrogen in urine eliminated during a fairly long period. Consequently only a very small dose (0.513 gram) was given on the 11th day (1st day of the period), but the dose was gradually increased until the 21st day, when 7.001 grams were given. By that time the purgative effect was so pronounced that we were forced to discontinue dosage. Otherwise the dog seemed perfectly normal. He was cheerful and showed no signs of distress.

THIRD PERIOD. *Intermediate period. Days 22-29; December 2-9.* The recovery of the dog from the diarrhea was almost immediate. On the 22d day diarrhea was most pronounced as an effect of dosage on the last day of the previous period, but on the 23d day the feces were only moderately diarrheal. On the 24th day they were almost normal. Thereafter in this period there were no diarrheal tendencies.

FOURTH PERIOD. *Influence of magnesium sulfate when injected subcutaneously. Days 30-41; December 10-21.* By the end of the preceding period the dog had practically returned to normal conditions. We next attempted to ascertain the influence of magnesium sulfate injected subcutaneously. We used very small amounts at first but gradually increased the daily dose until the general effects were so decided that it was obviously inadvisable to continue the treatment.

*Schedule of injections of magnesium sulfate. 30th day.* At 12:45 p.m., 0.01 gram dissolved in 1 cc. of water was injected on the right side. There were no appreciable effects. *31st day.* At 12:35 p.m., 0.025 gram dissolved in 1 cc. of water were injected on the left side. Again there were no particular effects. The feces for the day were a trifle softer than normal. *32d day.* At 12:50 p.m., 0.05 gram dissolved in 1 cc. of water were

## 90 Influence of Magnesium Sulfate on Metabolism

injected on the right side. The animal was uneasy after the injection, squirming about and rolling on his back. He defecated at 1 p.m. The feces were soft, clay-like. *33d day.* At 12:50 p.m., 0.1 gram dissolved in 2 cc. of water was injected near the shoulder on the left side. The feces had the consistency of putty and the urine was turbid. *34th day.* At 1:10 p.m., 0.2 gram dissolved in 1 cc. of water were injected on the right side. The feces were a trifle softer than on the day before. The turbidity of the urine was about the same. *35th day.* At 12:45 p.m., 0.4 gram dissolved in 2 cc. of water were injected on the right side. The feces were softer than on the previous day. The turbidity of the urine was also greater. *36th day.* At 1:05 p. m., 0.6 gram dissolved in 1.5 cc. of water were injected on the left side. The feces were partly soft and partly hard, and the turbidity of the urine was about the same as on the previous day. *37th day.* At 1 p.m., 0.6 gram dissolved in 1.5 cc. of water were injected on the right side. The dog was more decidedly affected than before. After the injection he was very restless at first and then became unusually sleepy. The feces were hard. The urine was very turbid and had considerable sediment. *38th day.* At 12:50 p.m., 1 gram dissolved in 2.5 cc. of water was injected on the left side. After the injection the dog was very uneasy and apprehensive at first, but then gradually became dull and drowsy and remained so until evening. The feces were hard and dry and the urine had a heavy sediment. *39th day.* At 12:40 p.m., 1 gram dissolved in 2.5 cc. of water was injected on the right side. At 4 p.m. another gram was injected on the left side. After the first injection the dog showed the symptoms exhibited on the day before, namely, at first he was very restless and apprehensive and then soon became drowsy. After the second injection he immediately assumed a recumbent position, then went to sleep and slept all afternoon and evening. On being awakened at 9:15 p.m., he urinated. The next morning there were signs of edema on the left side in the lumbar region and he was sore and stiff in his hind legs. The feces were dry and brittle. The urine had a heavy sediment. *40th day.* At 12:40 p.m., 5 grams dissolved in 8 cc. of water were injected on the left side. The dog became very drowsy within a couple of minutes and could not be persuaded to arise. He cuddled up in the corner of the cage and shivered as if he were cold. Occasionally he got up to urinate. The total amount of urine for the day was almost double the amount of water given with his food. The feces were very hard and brittle. Next morning the dog was bright and cheerful. *41st day.* At 12:50 p.m., 10 grams dissolved in 12 cc. of water were injected on the right side. The animal exhibited practically the same symptoms as those shown on the 40th day, and was even more sleepy. The sleepiness had not worn off by the next morning. The dog then acted as if in great pain when he was touched in the region of the last injection. His appetite for the first time was poor.

**FIFTH PERIOD.** *After period. Days 42-47; December 22-27.* During this period the dog was permitted to recover from the effects of the dosage. On the right side in the lumbar region, where the last injection was made,

a swelling gradually developed which opened on the 44th day, revealing a considerable area of necrosis. Sloughing was prompt and complete. No treatment of the wound was necessary, as the surface tended to become dry and healed rapidly. By the 47th day the dog seemed to be normal again in all respects with the exception of the sore on the side.

SIXTH PERIOD. *Supplementary period. Days 48-62; December 28, 1907-January 11, 1908.* The dog was kept under general observation for two weeks in order that possible "after effects" might be detected.

ANALYTIC RESULTS. The more important data in our records of the first metabolism experiment are given in Tables II and III.

DISCUSSION OF THE RESULTS. *Changes in the weight of the dog.* Examination of the data recorded in Table II shows that there was a gradual and continuous increase in weight during the fore period (1st), throughout the period during which magnesium sulfate was given *per os* (2d), during the intermediate period (3d), and during the first half of the period in which magnesium sulfate was administered *subcutaneously* (4th). During the second half of the latter period there was a rapid decrease of weight, due, undoubtedly, to the diuretic effect of the larger subcutaneous doses of magnesium sulfate. The decline in weight reached its lowest ebb on the last day of the period (4th) of subcutaneous injection. During the fifth or "after" period, the weight quickly rose, but did not quite return to the maximal point (10.70 in the 4th period) before the end of the supplementary period.

*Fluctuations in the daily volume of urine.* The amount of water in the food remained the same each day, yet, as an examination of Table II will show, the volume of urine fluctuated through quite a wide range. The minimal amount was 155 cc. on the 44th day, during the "after" period, when the animal was rapidly recovering from the effects of the diuresis of the subcutaneous injection period. The maximal amount was 716 cc. on the 40th day (next to the last day of the injection period). The relatively large doses of injected magnesium sulfate in the latter part of the fourth period appeared to exert a marked diuretic influence. In the fifth period there was a corresponding retention of water, and a consequent decrease of urine volume, as the weight of the animal rose.

The comparatively uniform daily volume of urine in the second period indicates that the doses of magnesium sulfate that were

## 92 Influence of Magnesium Sulfate on Metabolism

administered *per os* during that period had very little influence on the volume of urine. The slightly reduced average daily volume of urine for that period (Table III) is explained by the moderate diarrhea that resulted from the corresponding doses *per os*.

*Alterations of the specific gravity of the urine.* With a few exceptions the specific gravity of the urine showed little variation from the average except in the second and fifth periods, when the excreted proportions of magnesium sulfate were large, while at the same time the volumes of urine were at or below the average, or when the volumes of urine were particularly small while the nitrogen catabolism was comparatively extensive.

*Effects on the elimination of feces.* Aside from the fecal effects already noted, little can be said in this connection. Fairly large doses *per os* made the feces softer. In the early part of the injection period doses subcutaneously administered appeared to cause slight softening. This may have been a mere coincidence, however. The subsequent larger doses seemed to make the fecal masses more brittle than they were during the normal period. The average daily quantity of dry fecal matter increased slightly per period until the fourth, inclusive, then fell off rather decidedly during the last two periods.

*Effects on the elimination of total nitrogen of the urine.* The object of this experiment was primarily to ascertain the effect of magnesium sulfate on the partition of urinary nitrogen. Therefore we did not analyze the feces nor cast off hair in this experiment and consequently made no attempt to ascertain whether the animal was in nitrogenous equilibrium. Examination of Table III shows, however, that in the post-subcutaneous injection period (5th) the total amount of urinary nitrogen was considerably higher than in any of the other periods, and greater than the total quantity of ingested nitrogen. In the injection period (4th) the average daily elimination of urinary nitrogen was practically the same as during the previous period (3d) and less than the amount of ingested nitrogen. That the increased elimination of total nitrogen in the urine of the fifth period cannot be ascribed to the mere diuretic influence of the injected magnesium sulfate is evident from the fact that during that period (5th) in which the greatest elimination of nitrogen occurred the volumes of urine were the smallest of the experiment. During



the period in which magnesium sulfate was given *per os*, there was no increase in the total urinary nitrogen.

*Effects on the partition of the total urinary nitrogen.* The most noticeable effects on the distribution of urinary nitrogen among the products named at the head of Table III were (1) a fairly uniform absolute average daily excretion of *urea* but a striking decrease in its relative elimination, beginning with the period after administration of magnesium sulfate *per os* and continuing to the end of the experiment; and (2) cumulative increases in both the absolute and relative excretions of nitrogen in the forms of *allantoin*, *uric acid*, *purin bases* and *undetermined products*. There was a slight increase in the relative *urea* excretion in the second period, but it hardly deserves special attention. *Creatinin* elimination was fairly uniform throughout the experiment, although there were indications of slightly increased output, both absolute and relative, in the dosage periods. The *ammonia* nitrogen was exceptionally high during the period in which magnesium sulfate was given *per os* and again in the last (5th) period. It was low in the subcutaneous injection period. The anomalous results especially in the subcutaneous injection period were doubtless due to the sources of error referred to in the preceding paper.<sup>1</sup> *Ammonia* elimination appears to have been very perceptibly increased in the second period as the daily doses of magnesium sulfate *per os* were increased.

See the discussion on p. 118 for further reference to these results.

<sup>1</sup> Steel and Gies: This *journal*, this volume, p. 71.

# 94 Influence of Magnesium Sulfate on Metabolism

TABLE II.

*Some of the daily records of the first experiment.  
The first metabolism experiment.*

FIRST PERIOD. *Normal conditions. Days 1-10; November 11-20, 1907.*

Day No.	Body weight.	Dose of $MgSO_4$ .	URINE.						FECES.
			Volume.	Specific gravity.	Total nitrogen.	Urea nitrogen.	Ammonia nitrogen.	Creatinin nitrogen.	
	<i>kilos.</i>	<i>grams.</i>	<i>cc.</i>	<i>10xx.</i>	<i>grams.</i>	<i>grams.</i>	<i>grams.</i>	<i>grams.</i>	<i>grams.</i>
1	10.19		280	15			0.123		21.0
2	10.17		478	13	10.224	8.482	0.152	0.228	30.4
3	10.14		380	14			0.182		54.7
4	10.13		390	13	9.964	8.686	0.130	0.247	28.4
5	10.09		418	13			0.142		31.5
6	10.15		290	15	9.662	8.398	0.124	0.227	41.4
7	10.26		355	15			0.132		0
8	10.20		352	16	10.376	9.134	0.133	0.226	44.2
9	10.24		360	13			0.094*		25.4
10	10.25		380	16	9.800	8.657	0.175	0.228	13.9

\* This amount is small, but was correctly determined.

SECOND PERIOD. *Effect of magnesium sulfate administered per os.*

*Days 11-21; November 21-December 1.*

11	10.25	0.513	342	15			0.158		46.4
12	10.29	0.504	350	15	9.662	8.467	0.122	0.229	0
13	10.34	0.999	360	15			0.127		17.7
14	10.44	1.506	310	18	9.789	8.662	0.171	0.219	21.2
15	10.37	2.001	400	15			0.219		55.7
16	10.33	2.499	382	16	10.298	9.089	0.221	0.256	39.0
17	10.40	3.000	366	17			0.259		0
18	10.42	3.999	348	18	10.278	8.975	0.269	0.239	47.2
19	10.42	5.001	340	18			0.279		40.5
20	10.44	6.006	360	20			0.307		23.2
21	10.46	7.001	325	22	14.895	12.866	0.284	0.379	29.5

TABLE II—CONTINUED.

THIRD PERIOD. *Intermediate period. Days 22-29; December 2-9.*

Day No.	Body weight.	Dose MgSO <sub>4</sub> .	URINE.						FECES.
			Volume.	Specific gravity.	Total nitrogen.	Urea nitrogen.	Ammonia nitrogen.	Creatinin nitrogen.	
	kilos.	grams.	cc.	10xx	grams.	grams.	gram.	gram.	grams.
22	10.40		388	15			0.253		26.1
23	10.39		350	15	12.169	10.481	0.200	0.236	37.9
24	10.48		300	15			0.132		22.1
25	10.45		330	16	10.812	9.268	0.138	0.186	31.1
26	10.42		412	13			0.101		34.6
27	10.47		314	16	10.270	9.201	0.124	0.223	23.0
28	10.55		290	18			0.135		29.2
29	10.54		385	14	10.733	8.368	0.134	0.200	29.1

FOURTH PERIOD. *Effects of magnesium sulfate injected subcutaneously. Days 30-41; December 10-21.*

30	10.66	0.010	268	16			0.152		13.9
31	10.61	0.025	348	16	9.747	7.582	0.165	0.227	20.4
32	10.70	0.050	306	16			0.136		37.2
33	10.66	0.100	310	16	9.806	7.999	0.142	0.232	27.0
34	10.67	0.200	340	17			0.124		44.2
35	10.70	0.400	270	19	10.025	7.741	0.155	0.243	35.1
36	10.60	0.600	420	14			0.125		36.9
37	10.64	0.600	230	24	9.712	8.356	0.149	0.213	18.1
38	10.61	1.000	498	15			0.222		33.2
39	10.50	2.000	430	18	11.829	9.378	0.165	0.263	33.3
40	10.15	5.000	716	15			0.112		31.8
41	10.03	10.000	479	24	14.329	7.698	0.149	0.419*	26.5

FIFTH PERIOD. *After period. Days 42-47; December 22-27.*

42	10.20		225	33			0.175		11.4
43	10.30		224	38	16.082	10.097	0.374	0.264	33.2
44	10.42		155	37			0.161		41.2
45	10.37		300	25	15.054	10.009	0.315	0.223	42.9
46	10.41		260	23			0.286		20.4
47	10.52		290	21	12.224	6.827	0.219	0.203	21.0

\* This amount is large, but was correctly determined.

# 96 Influence of Magnesium Sulfate on Metabolism

TABLE II—CONTINUED.

SIXTH PERIOD—*Supplementary period.*

*Days 48-62; December 28, 1907 to January 11, 1908*

Number of the day.....	48	49	50	51	52	53*	54	55
Body weight (kilos).....	10.50	10.54	10.50	10.52	10.49	10.42	10.44	10.50
Urine: Volume (cc.) .....	340	358	376	312	420	360	345	314
Specific gravity (10xx)...	16	17	15	16	13	17	16	17
Feces: weight (grams).....	36.4	18.5	33.0	25.1	31.8	38.6	15.2	15.3
Number of the day.....	56	57	58	59	60	61	62	
Body weight (kilos).....	10.41	10.47	10.50	10.53	10.54	10.59	10.61	
Urine: Volume (cc.) .....	363	420	310	334	342	330	350	
Specific gravity (10xx).....	17	16	18	17	17	18	15	
Feces: weight (grams).....	37.0	23.4	21.3	54.2	14.0	21.9	26.8	

\*The total nitrogen was 29.90 grams for days 48 to 53, inclusive, or an average of 4.983 grams per day.

TABLE III.  
FIRST EXPERIMENT. Summary of analytic totals for each period.

PERIOD.		FOOD.		URINE.										FECES.	
Condition.		Total nitrogen, nitrogen.		Nitrogen in the form of—										Dry weight.	
No.		grams.	grams.	Urea.	Ammonia.	Creatinin.	Allantoin.	Uric acid.	Purin bases.	Undeter- mined.	Volume.	cc.	grams.		
	I Normal	61.540	50.026	43.357	1.387	1.156	0.358	0.194	0.066	3.508	3684	200.0	200.0		
	II MgSO <sub>4</sub> per os	72.314	54.922	48.050	2.416	1.322	0.340	0.212	0.126	2.447	3885	320.4	320.4		
	III Intermediate	52.592	43.084	37.318	1.217	0.845	0.298	0.187	0.068	4.144	2769	233.1	233.1		
	IV MgSO <sub>4</sub> subcutaneous	77.748	65.448	48.754	1.766	1.507	0.686	0.350	0.246	12.010	4014	357.6	357.6		
	V After	39.024	43.360	26.933	1.530	0.600	0.540	0.760	0.233	12.074	1454	170.1	170.1		
	VI Supplementary	60.060	20.90*								5274	412.4	412.4		
Daily averages.															
	I Normal	6.154	5.003	4.336	0.130	0.116	0.036	0.010	0.007	0.351	368	20.09	20.09		
	II MgSO <sub>4</sub> per os	6.574	4.993	4.369	0.219	0.120	0.031	0.019	0.011	0.222	353	20.13	20.13		
	III Intermediate	6.574	5.498	4.065	0.152	0.106	0.037	0.023	0.008	0.518	346	20.13	20.13		
	IV MgSO <sub>4</sub> subcutaneous	6.479	5.454	4.063	0.149	0.133	0.057	0.020	0.021	1.002	364	20.80	20.80		
	V After	6.004	7.227	4.489	0.255	0.115	0.090	0.127	0.039	2.112	342	28.35	28.35		
	VI Supplementary	6.004	4.063†								352	27.50	27.50		
Relation to the total nitrogen.															
	I Normal			86.06	2.58	2.32	0.72	0.38	0.14	7.01					
	II MgSO <sub>4</sub> per os			87.50	4.38	2.40	0.92	0.38	0.22	4.45					
	III Intermediate			85.01	2.76	1.92	0.67	0.42	0.14	9.42					
	IV MgSO <sub>4</sub> subcutaneous			74.49	2.71	2.44	1.04	0.53	0.38	18.37					
	V After			62.11	3.52	1.50	1.23	1.75	0.54	20.22					

\* Total quantity for the first six days (48-53).

† Average amount for the first six days (48-53).

## 98 Influence of Magnesium Sulfate on Metabolism

### IV. SECOND EXPERIMENT. DOES BONE ASH IN THE DIET OFFSET THE CATHARTIC INFLUENCE OF MAGNESIUM SULFATE GIVEN WITH THE FOOD?

It was thought that possibly the calcium of the bone ash in the food exerted a neutralizing effect on the influence of magnesium. In view of the fact, however, that calcium salts do not appear to be appreciably absorbed from bone ash under the conditions of our experiments,<sup>1</sup> the possibility of a suspension of magnesium influences in the manner indicated was rather remote. Nevertheless, before proceeding farther, we made a direct test of the matter, and attacked the problem at the point where results, if any were attainable, would most likely be revealed. It is a fair assumption, we think, that if bone ash in the food has no effect on the catharsis induced by accompanying doses of magnesium sulfate *per os*, it is likewise without influence on the behavior of magnesium sulfate administered subcutaneously or intravenously.

The animal selected for this experiment was a plump, vigorous, short haired male dog, weighing about 8 kilos. The daily diet was similar to that of the first experiment and contained the following proportions: Hashed lean meat, 120 grams; cracker meal, 32 grams; lard, 24 grams; water, 260 cc. The food was given daily at 11:30 a.m.

**FIRST PERIOD.** *Without magnesium sulfate and without bone ash.* Days 1-9; December 1-9, 1907. The dog was kept on the above-mentioned diet for nine days, without magnesium sulfate and *without bone ash*, during which period the soft, black feces exhibited the usual qualities of the excrementitious matter from the bowels of dogs on a diet consisting largely of meat. Defecations occurred only once about every third day.

**SECOND PERIOD.** *Influence of bone ash on the cathartic effect of magnesium sulfate.* Days 10-21; December 10-21. In this period the animal was given daily with the food a gradually increasing dose of magnesium sulfate. *without bone ash*, until decided diarrhea occurred. The dog was then kept on this dose *without bone ash* for a few days, during which time the diarrhea was fully maintained. When we were certain that the daily dose of magnesium sulfate would invariably produce diarrhea, 15 grams of bone ash were added to the daily food. The appended summary presents the plan of treatment and the results in some detail (Table IV).

*Plan of treatment with magnesium sulfate. 10th to 17th days, inclusive; magnesium sulfate without bone ash. 10th day (first day of dosage). Dose:*

---

<sup>1</sup> Steel and Gies: *Amer. Journ. of Physiol.*, xx, p. 343, 1907.

0.501 gram. The dog did not defecate. *11th day.* Dose: 1.002 grams. No feces. *12th day.* Dose: 2.001 grams. No feces. *13th day.* Dose: 3.003 grams. The dog defecated. The black fecal mass was formed but quite soft. *14th day.* Dose: 4.005 grams. The black fecal mass was unformed and like thick mud in consistency. *15th to 17th day.* Daily dose: 4 grams. Marked diarrhea occurred daily.

Eighteenth to 25th days, inclusive; *magnesium sulfate with bone ash.* *18th day.* Dose: 4 grams with 15 grams of bone ash. The dog defecated at about 3 p.m. The feces were thin diarrheal discharges like those of the past few days. At 10 a.m. shortly before the end of the day of record, the dog defecated again. This time the fecal discharge was a gray mass, which although unformed, was somewhat thicker than the feces eliminated during the 15th-17th days. It appeared to be in effect the usual diarrheal mass made grayish and thicker by the mechanical admixture with the bone ash. *19th to 21st days.* Daily dose: 4 grams with 15 grams of bone ash. The dog defecated daily. Each discharge was of approximately the same consistency as that of the second elimination on the 18th day. The degree of diarrhea was practically the same as in the first experiment under similar conditions of dosage.

These results make it evident that bone ash in the diet will not appreciably offset the cathartic effect of magnesium sulfate except in a purely mechanical way.

THIRD PERIOD. *With bone ash, but without magnesium sulfate.* Days 22-23; December 22-23. No magnesium sulfate was administered, but the daily quantity of bone ash (15 grams) was continued. The recovery from the cathartic effect was almost immediate. On the 23d day the feces were entirely devoid of diarrheal consistence.

(This animal was used again in the special experiment described in the footnote on p. 104.)

TABLE IV.

*Some of the daily records of the second experiment.*I. FIRST PERIOD. *Normal conditions. Days 1-9; December 1-9.*

	1	2	3	4	5	6	7	8	9
Number of the day.....									
Body weight (kilos).....	8.03	8.04	8.00	8.05	8.10	8.05	8.00	8.03	7.97
Urine: Volume (cc.).....	251	334	280	250	241	285	336	260	325
Specific gravity(10xx).....	18	14	16	15	17	15	13	15	15

II. SECOND PERIOD. *Influence of bone ash on the cathartic effect of magnesium sulfate. Days 10-21; December 10-21.*

	10	11	12	13	14	15	16	17	18	19	20	21
Number of the day.....												
Body weight (kilos).....	7.98	8.00	7.98	8.02	8.00	8.03	7.98	7.97	7.80	7.87	7.76	7.83
MgSO <sub>4</sub> (grams).....	0.501	1.002	2.001	3.003	4.005	4.004	4.003	4.001	4.005	4.001	3.999	4.003
Bone ash (grams).....									15.00	15.00	15.00	15.00
Urine: Volume (cc.).....	370	325	365	160	285	200	402	218	395	215	380	250
Sp. gr. (10xx).....	10	14	11	30	19	25	15	22	15	23	13	23

III. THIRD PERIOD. *After period.**Days 22-23; December 22-23.*

Number of the day.	Body Weight.	Bone Ash.	URINE.	
			Volume.	Specific Gravity.
			cc.	10xx
22	kilos.	grams.	302	17
23	7.70	15.00	194	20
	7.83	15.00		



V. THIRD EXPERIMENT. A DETERMINATION OF THE CONDITIONS MOST FAVORABLE FOR INTRAVENOUS INJECTION OF LARGE DOSES OF MAGNESIUM SULFATE INTO DOGS.

Before subjecting the dog of the fourth experiment (p. 102) to the influence of an intravenous injection of magnesium sulfate, we endeavored, in a preliminary trial experiment, to ascertain the procedure that could be followed to the greatest advantage in an effort to administer large doses directly into the circulation of a dog. The following abstract from the protocol of that experiment confirms the observation by Meltzer and Auer<sup>1</sup> that large doses of magnesium sulfate may be introduced intravenously provided injection is conducted very slowly.

The dog used in this experiment weighed about 10 kilos and had been kept on a diet very similar to that given the animal of the first metabolism experiment. Ten grams of magnesium sulfate, dissolved in 100 cc. of water, were injected into a saphenous vein, after anesthetizing locally with cocaine (1 cc. of a 2 per cent solution) at 4:21 p.m. A summary of operations and observations is appended.

4:22 p.m. Injection of the magnesium sulfate solution was started from a burette at the rate of about 1 cc. in 35 seconds. This rate was not increased but occasionally was decreased, as the effects on respiration became more decided. Among the early effects was a marked gastric disturbance, though vomiting did not occur. 5:00. Much salivation, augmented nasal secretion, and watering of the eyes. 5:10. Anesthetic effect quite marked. 5:25. Respiration weak. Reflex responses slight. 5:50. Profoundly anesthetized. 5:53. The last of the solution (100 cc.) injected. The animal was placed in a cage after rapid suturing of the skin at the site of operation. 5:58. The dog tried unsuccessfully to stand up. 6:02. Got up but was too weak to remain on his feet. 6:15. Arose and wagged his tail. Able to walk without difficulty. 6:20. Recumbent and going to sleep. 10:25. The dog urinated, and after that seemed almost normal again. During the first 24 hours after the injection there was diuresis. The urine was also exceedingly turbid and soon yielded a heavy sediment. The first samples of urine after the injection were very much paler in color than the portions eliminated previous to the injection. On the next day the dog seemed to have entirely recovered from the effects of the treatment. The anesthesia seemingly had no unfavorable sequellæ.

<sup>1</sup> Meltzer and Auer: *Amer. Journ. of Physiol.* xv, p. 389, 1906.

# 102 Influence of Magnesium Sulfate on Metabolism

## VI. FOURTH EXPERIMENT. SECOND METABOLISM EXPERIMENT.

The general conditions of the first metabolism experiment were duplicated in this experiment. There was also a study of intravenous effects.

The dog used in this experiment was a healthy, short haired female, weighing about 10.6 kilos. The diet was practically the same as that of the dog in the first experiment. This dog received 25 cc. more water per day than the first.

TABLE V.  
*Composition of the daily diet.*

INGREDIENTS.	HASHED LEAN BEEF.			CRACKER MEAL.	LARD	BONE ASH.	WATER.
Days .....	1-14	15-45	46-73	1-73			
	grams.	grams.	grams.	grams.	grams.	grams.	cc.
Daily amounts.....	150	150	150	40	30	20	375
Nitrogen.....	5.34	5.55	5.43	0.651	0.008	0.006	

**PREPARATORY PERIOD.** The dog was kept on the above-mentioned diet for nine days, before any analyses were made, in order to accustom her to the cage and diet. In four days her weight increased from 10.56 kilos to 10.72 kilos and then remained at about 10.70 kilos during the next five days. The preparatory period ended on February 17, 1908. On that day, at 11:30 a.m., the accumulation of analytic data was begun. The experiment was continued uninterruptedly until April 30 (73d day) and was divided into eleven periods of different lengths and conditions as follows:

**FIRST PERIOD. Normal conditions. Days 1-7; February 18-24, 1908.** During this period the weight of the dog remained remarkably constant. She seemed to be perfectly satisfied with her surroundings.

**SECOND PERIOD. Influence of magnesium sulfate administered per os. Days 8-14; February 25-March 2.** With our previous experience in mind we began the treatment with a fairly large dose.

**Schedule of administrations of magnesium sulfate.** With the food of the 8th day (1st day of the 2d period), 3.001 grams were given. No effects could be observed. There was no defecation. 9th day. 3.998 grams were given. The feces were normal. 10th day. 5.002 grams were given. The feces were very soft. The diarrhea was so pronounced that the dose was not increased, and the animal was kept on this daily amount on the succeeding (11-14) days. On the 14th day (last of the period) the feces were so soft that they ran into the urine receiver, which, however, had no urine in it at the time. The diarrheal effect of 5 grams was greater in this dog than that of 7 grams in the first dog. The average volume of urine was also considerably lower.

THIRD PERIOD. *First intermediate period. Days 15-22; March 3-10.* The recovery from the diarrhea was quite rapid. On the 15th day (1st of this period) the animal did not defecate. On the 16th day, however, defecation occurred twice; the first mass was like stiff clay, the second discharge was hard and brittle. The urine did not show a marked tendency to increase in volume until the 19th day (5th of the period). After that the urinary volume was relatively large.

FOURTH PERIOD. *Influence of magnesium sulfate when injected subcutaneously. Days 23-26; March 11-14.* A 40 per cent solution of magnesium sulfate was used. Each day's portion was diluted to 20 cc. before injection.

*Schedule of injections of magnesium sulfate.* On the 23d day (1st of the period), at 12:50 p.m., 1 gram was injected on the right side. The initial effect was restlessness, followed by sleepiness until about 11 p.m., when 300 cc. of turbid urine were eliminated, after which the animal appeared to be more lively. On the following morning, she was bright and cheerful. The feces were normal. 24th day. At 12:55 p.m., we injected 2 grams on the left side. At 1:07 she was very restless and apprehensive; at 1:15 drowsy. She slept all the afternoon and at 8:15 p.m. urinated 300 cc. of very turbid urine; then she cuddled down in the corner of the cage as if trying to keep warm, although the room temperature was normal. At 9 a.m. she was lively and cheerful again. The feces were normal. 25th day. At 12:57 p.m., 4 grams were injected in the right side. As soon as the injection had been made, the dog rolled on her back and bit viciously for a moment or two at the place injected, then she cuddled down in the corner of the cage and could not easily be aroused. She slept all the afternoon and evening. At 11:15 p.m. she urinated 315 cc.; afterwards she was lively and more cheerful. The urine had a very heavy sediment and was alkaline to litmus. On the following morning an abundance of fine triple phosphate crystals had deposited on the sides of the vessel containing the urine. No feces were passed. 26th day. At 12:55 p.m., 8 grams were injected on the left side. At 1:10 p.m. she was lying on the bottom of the cage, panting and breathing rapidly with her tongue protruding. At 1:20 p.m. she was perfectly motionless as if in heavy slumber; her respiration was deep and slow. She slept all the afternoon, refusing to arise when spoken to. The next morning marked depression was a conspicuous symptom. The feces were very hard and dry and were passed with considerable difficulty. There was decided swelling on the left side at the place where the last injection was made, and the dog appeared to suffer considerable pain as a consequence of it.

FIFTH PERIOD. *Second intermediate (post-subcutaneous injection) period. Days 27-31; March 15-19.* On the first day of this period (27th day) the dog was very much depressed, and had to be coaxed to eat, whereas theretofore, she had always been eager for her food. She looked very apprehensive, shivered a great deal and was sleepy. Towards evening she was much brighter and more responsive when spoken to. The urine had a very heavy white sediment. The next day (28th) the animal ate

her food with relish. The necrotic mass on the left side had opened during the night. The opening was about three inches long and one and a half inches wide. The wound was enlarged to that size by the dog in her endeavor to remove dead material.<sup>1</sup> All of the latter was evidently swallowed by the animal, for none of it could be found in the cage. Recovery was rapid. Return to normal conditions was complete, before the beginning of the next period.

**SIXTH PERIOD.** *Influence of magnesium sulfate injected intravenously. Days 32-36; March 20-24.* With the results of the fourth experiment to guide us, we proceeded with an intravenous injection as follows: Ten grams of magnesium sulfate were dissolved in 100 cc. of water, and injected into a saphenous vein, after anesthetizing locally with cocaine (1 cc. of a 2 per cent solution). A summary of operations and observations is appended:

*Schedule of operations. 32d day.* At 3:17 p.m., the injection was started at the rate of about 1 cc. in 50 seconds, and maintained at about that rate throughout the entire procedure. 3:24. The dog began to show effects by dropping her head and gagging. Breathing was irregular. 3:28. The dog vomited. (The vomit was fully recovered and fed to her at 10 p.m.) 3:35. Breathing was very rapid. Anesthesia increased.

---

<sup>1</sup> The dogs of both metabolism experiments were affected in the same way locally by the subcutaneous injections of magnesium sulfate, but necrosis and sloughing occurred more promptly in the dog of the second *metabolism* experiment. The following special experiment, on the dog used in the second experiment (p. 98), may be cited as giving additional evidence of the marked local effects of magnesium sulfate when introduced under the skin in relatively large doses.

**FIRST PERIOD.** *Normal conditions. Days 1-2; December 22-23, 1907.* (See Table VI.)

**SECOND PERIOD.** *Local influence of subcutaneous doses of magnesium sulfate. Days 1-2; December 24-25, 1907. 1st day.* At 12:40 p.m., 8 grams, dissolved in 10 cc. of water, were injected subcutaneously on the right side. The dog exhibited the same symptoms that the other dogs showed under similar circumstances, i. e., at first he was very restless and then gradually became drowsy and slept all the afternoon. Late in the evening, slight edema was noticeable a few inches below the point of injection. *2d day.* At 1 p.m., 10 grams, dissolved in 12 cc. of water, were injected on the left side. The symptoms exhibited the day before were again elicited, the only difference being that the anesthetic effect was a little more pronounced. The next morning, at 10 o'clock, the animal had apparently entirely recovered from the anesthetic effect. The edema on the right side had disappeared, but on the left side, a few inches below the place of injection, there was considerable swelling and general soreness. The urine was pale in color and deposited a heavy sediment of fine triple phosphate crystals. The feces were dry and brittle.

**THIRD PERIOD.** *After period. Days 3-14; December 26, 1907-January 6, 1908.* The dog rapidly recovered from the general soreness.

4:17. Breathing weakly. 4:21. Deep anesthesia. No conjunctival reflex. 4:26. Anesthesia profound. Respiration almost entirely suspended. 4:27. Injection stopped. By this time exactly 80 cc. had run in, which contained 8 grams of the magnesium compound. 4:28. Breathing had nearly stopped. Respiration was artificially aided for several minutes by appropriate foreleg and tongue movements and chest compressions. 4:30. Conjunctival reflex sluggish. 4:35. Breathing more regular. 4:40. Decided conjunctival reflex. 4:53. After stitching the wound the animal was placed in a cage. She immediately arose but was too weak to stand. Was sleepy. 5:45. Awake and bright, very responsive when spoken to. 7:30. Lies cuddled up in a corner of the cage shivering as if cold. Room temperature normal. When spoken to, she got up. She soon urinated. The urine was very light colored. At 10 o'clock on the following morning, the animal was active and apparently had recovered fully from the anesthetic effects.

The swelling on the *left* side opened on the 4th day. Sloughing was relatively extensive and an unsightly wound resulted. Healing was in rapid progress on the 14th day, when observation was discontinued.

TABLE VI.  
SPECIAL EXPERIMENT.

I. FIRST PERIOD. <i>Normal Conditions.</i> Days 1-2; December 22-23, 1907.			II. SECOND PERIOD. <i>Local influence</i> <i>of magnesium sulfate injected</i> <i>subcutaneously.</i> Days 3-4; December 24-25, 1907.		
Number of day (period)...	1	2	Number of day (period)...	3	4
Body weight (kilos).....	7.70	7.83	Body weight (kilos).....	7.82	7.68
Dose of MgSO <sub>4</sub> (grams)...			Dose of MgSO <sub>4</sub> (grams)...	8.00	10.00
Urine: Volume (cc.).....	302	194	Urine: Volume (cc.).....	317	405
Specific gravity (10xx)...	17	20	Specific gravity (10xx)...	27	24

III. THIRD PERIOD. *After period.*  
Days 5-16; December 26, 1907-January 6, 1908.

No. of day (period)...	5	6	7	8	9	10	11	12	13	14	15	16
Body weight (kilos).....	7.65	7.61	7.72	7.82	7.63	7.80	7.72	7.80	7.78	7.93	8.00	8.04
Urine: Volume (cc.).....	280	268	185	185	356	168	304	210	318	188	225	267
Specific gravity (10xx)...	21		23	25	14	25	14	17	17	18	17	20

The different degrees in the effects of the intravenous injections in the two dogs was quite noticeable. In the animal of the third experiment the anesthesia developed gradually and was not nearly fatal, even when 10 grams had been injected, whereas in the dog of this experiment the effect was not nearly so strong at first, but came on quickly and was nearly fatal when only 8 grams had been injected. The rate of injection was somewhat slower in this experiment than in the third experiment. In the latter experiment the dog did not vomit, although gastric disturbance was noticeable. In this experiment, as recorded above, vomiting occurred soon after injection was started. There was also a marked difference in the nature of the urine from the two animals on the night and day following the injections. The first dog excreted urine containing a heavy sediment. The second eliminated a larger volume, containing only a very trifling amount of sediment. As in the case of the animal of the third experiment, the dog in this one recovered rapidly and showed no lasting effects of the intravenous injection. The feces were normal at each elimination, certainly not softer than usual.

**SEVENTH PERIOD.** *Second intermediate period. Days 37-45; March 25-April 2.* During this part of the experiment the general condition of the animal was noted. Normal conditions appeared to prevail, as recovery from the previous treatment had been very prompt. The ammonia in the urine was determined daily and the total amount of nitrogen in the urine of the whole period of nine days was ascertained. The dog increased in weight from 10.53 to 10.68 kilos. The average volume of urine was normal.

**EIGHTH PERIOD.** *New Normal period. Days 46-51; April 3 to 8.* In this period the general condition of the dog was noted and analytic data collected as in an ordinary fore period. In anticipation of further observations on the effects of intravenous injections, the usual analytic routine was followed in detail. The weight of the animal continued to rise a little and was highest on the 51st day (the last day of the period). The average volume of urine was a trifle higher than it had been in the previous period. By the end of this period, the wound caused by the subcutaneous injection on the 26th day (the last day of the fourth period) had almost entirely healed.

**NINTH PERIOD.** *Second intravenous injection of magnesium sulfate. Days 52-57; April 9-14.* We attempted to duplicate as closely as possible all conditions attending the first intravenous injection in this experiment (6th period, 32d day). Accordingly, 10 grams of magnesium sulfate were dissolved in 100 cc. of water, and injected into a saphenous vein, after anesthetizing locally with cocaine (1 cc. of a 2 per cent solution). A summary of the operations and observations is appended.

*Schedule of operations. 52d day.* At 3:25 p.m., the injection was started at the rate of about 1 cc. in 47 seconds, and maintained at about that rate throughout the entire procedure. 3:30. The dog begins to show effects by panting and breathing rapidly. 3:34. The heart is beating very rapidly. The dog showed considerable signs of gastric disturbance but did not vomit. 4:00. Shows signs of increasing anesthesia.

Heart beats slowly. Respiration weak. 4:10. Anesthesia increasing rapidly. Conjunctival reflex sluggish. 4:18. No conjunctival reflex. Breathing in gasps. 4:23. Respiration almost entirely inhibited. 4:24. Profound anesthesia. Injection stopped. By this time exactly 70 cc. had run in, which volume contained 7 grams of magnesium sulfate. 4:30. Respiration more regular. 4:34. Slight conjunctival reflex. 4:35. After closure of the wound, the animal was placed in the cage, where she remained practically motionless for about 20 minutes and then stood up, but seemed very weak. In a minute she lay down again and went to sleep. While sleeping one of her paws twitched spasmodically. 9:15. She urinated 600 cc. of very light, clear urine; afterwards was bright and cheerful. Later in the night she urinated 65 cc. of urine. This was likewise very clear. There was no sediment. The specific gravity of the first portion eliminated (the 600 cc.) was 1016 and the reaction was amphoteric to litmus. The specific gravity of the second portion (65 cc.) was 1023, and its reaction decidedly acid. When the two portions were united the specific gravity was 1017 and the reaction faintly acid to litmus. The dog's weight fell considerably as was to be expected, from the fact that a particularly large volume of urine had been eliminated. The weight of the dog steadily rose again, however, and by the 57th day (last of the period) was at the normal point. No lasting effects of the injection were noticeable. The feces at each elimination were normal, perhaps drier and more brittle than usual.

**TENTH PERIOD.** *Third intermediate period. Days 58-63; April 15-20.* Only the general condition of the animal was noted in this period. Her weight remained very constant, between 10.76 and 10.79 kilos. The volume of urine was comparatively large and the specific gravity uniform.

**ELEVENTH PERIOD.** *Influence of an intravenous injection of magnesium sulfate on the magnesium content of the feces. Days 64-73; April 21-30.* Before discontinuing the experiment we concluded to conduct a final intravenous injection with a view primarily of determining some facts regarding the excretion of magnesium in the feces after introduction of a soluble salt into the circulation. Special care was taken to prevent contact between urine and feces. Ten grams of magnesium sulfate were dissolved in 100 cc. of water and the solution injected into a saphenous vein after locally anesthetizing with cocaine (1 cc. of a 2 per cent solution).

*Schedule of operations. 64th day.* At 4:05 p.m., the injection was started at the rate of about 1 cc. in 36 seconds, and maintained at approximately that rate throughout the entire procedure. 4:18. The dog showed marked signs of gastric disturbance. 4:20. Vomited. 4:24. Heart beating very rapidly. 4:26. Increased signs of anesthesia. 4:29. Heart fluttering. Conjunctival reflex sluggish. 4:39. Marked abdominal respiration and increased fluttering of the heart. 4:41. No conjunctival reflex. 4:50. Profound anesthesia. Respiration almost entirely suspended. 4:55. Injection stopped. By this time exactly 85 cc. had run in, which volume contained 8.5 grams of magnesium sulfate. Recovery was prompt. At 5:10 after stitching the lips of the wound the dog was put

in a cage. At 5:18 the dog stood up in the cage and began licking the wound; after a moment she lay down again and went to sleep. As in the former experience one of the paws twitched spasmodically for some time at this stage. 9:30. The dog was bright and cheerful, and urinated 365 cc. of very clear urine. The vomit was then offered. She ate it all very readily. Late at night she urinated 190 cc. of very turbid urine. The reaction of the first portion eliminated (the 365 cc.) was alkaline to litmus. The reaction of the second portion (the 190 cc.) was amphoteric to litmus. When the two portions were united the reaction was faintly alkaline. During most of the period the feces were analyzed daily for magnesium.

**ANALYTIC RESULTS.** The essential data of this experiment are presented in Tables VII-IX.

**DISCUSSION OF THE RESULTS.** *Changes in the weight of the dog.* The weight of the dog was comparatively uniform until the fifth period. On the 28th day (the second day of the post-subcutaneous injection period there was a noticeable fall in weight. On the next day the decrease was unusual. The decline continued for a few days and reached the lowest point in the experiment on the 32d day (first day of the first intravenous injection period). The weight then very slowly returned by the end of the eighth period (51st day) to that at the beginning of the experiment. It fell sharply immediately after each of the subsequent intravenous injections but rapidly returned to the normal each time (9th and 11th periods).

Administration *per os* was without effect on the weight of the dog, but there was a marked decline in weight in the period immediately after the subcutaneous injection. After the intravenous injections there was always a sharp decrease in weight which was most pronounced within twenty-four hours after the treatment, and which was not cumulative, as appeared to be the case after subcutaneous administration.

*Fluctuations in the daily volume of urine.* The daily volume of urine fluctuated between 270 cc. on the second day (33d) of the first intravenous injection period (6th) and 665 cc. on the first day (52d) of the second intravenous injection period (9th). The average daily quantity was relatively high during the period (5th) succeeding the subcutaneous injection and during the last period. Administration *per os* appeared to cause perceptibly decreased volume of urine, evidently in compensation for the



loss of water in the diarrheal stools. The largest daily volumes were eliminated on the days of intravenous injection.

*Alterations of the specific gravity of the urine.* The extreme registrations of specific gravity of the urine were 1012 in the opening period and 1038 in the fourth period (subcutaneous injection period). The former value pertains to a relatively high volume and the latter to a comparatively small one, so that the figures quoted are not particularly significant. In a general way, specific gravity was higher after administration of magnesium sulfate *per os* than during the normal period. The reduced volume of urine during the second period fully explains that fact. The higher specific gravity of the volumes excreted shortly after subcutaneous injection (4th period) were seemingly due chiefly to the eliminated magnesium sulfate. The fluctuations of specific gravity during the last seven periods were slight. Few of the figures went above the normal ones. Although the specific gravity of the urine eliminated on the first day of each intravenous injection period was somewhat above normal, the diuresis that immediately resulted after such injections prevented the figures for specific gravity from registering at the higher points they would otherwise have attained, because of the prompt elimination of the injected magnesium sulfate.

*Effects on the elimination of feces.* Administration of magnesium sulfate *per os* made the feces softer, as has already been noted. The injections, on the other hand had no effect at all on their consistency or else actually rendered the fecal discharges drier and more brittle. In one case after intravenous injection, as has already been noted, the feces were passed with great difficulty, whether from inhibition of the normal muscular movements or because of particular dryness alone, can hardly be said.

The average daily quantity of *dry* fecal matter was somewhat greater than normal in the second and all subsequent periods. In a general way the figures for dry fecal matter indicate specially increased daily output promptly after administration of magnesium sulfate *per os*, subcutaneously or intravenously. In only one case was the increased amount of dry fecal matter accompanied by an increased content of nitrogen in the feces. The average daily elimination of nitrogen in the feces per period was quite uniform throughout the entire experiment, although slightly subnormal in all but the post-subcutaneous and first intravenous injection periods.

*Effect on the total nitrogen of the excreta.* The data in Table IX show that our animal was not in nitrogenous equilibrium at the beginning of the experiment. The retention balance of nitrogen diminished per period until the fourth (subcutaneous injection period) when the dog was in practical nitrogenous equilibrium. In the fifth or post-subcutaneous injection period, perhaps as an "after effect," there was a large minus balance. The development and opening of the abscess may have influenced the outcome. Retention was the rule again in the subsequent periods, with progressive period increase toward the highest plus balance in the seventh period (period after the first intravenous injection) and regular recession to a small plus balance in the last period.

These results might be assumed to indicate that the doses of magnesium sulfate increased the total elimination of nitrogen, but the last four plus balances, those in the sixth and seventh periods particularly, make such an inference difficult to maintain. The explanation of the outcome in this regard is not at all clear.

*Effects on the elimination of total nitrogen in the urine.* In almost perfect harmony with the trend of the period balances, as given in Table VIII, there was a progressive increase in the daily average amount of urinary nitrogen to the maximum excretion in the fifth period (post-subcutaneous injection period), succeeding which there was cumulatively decreased elimination until the last or second intravenous injection period, in which there was again a comparative increase. The significance of these results is not at all clear, although the development and opening of the abscess probably affected the data appreciably. In this experiment, and in the first *metabolism* experiment, the maximal period outputs of urinary nitrogen occurred during the fifth or post-subcutaneous injection period. In the preceding *metabolism* experiment the maximal excretion of urinary total nitrogen was associated with the smallest average daily volume of urine for the period. In this experiment, on the other hand, the maximal elimination of urinary total nitrogen occurred during the period in which the largest volumes of urine were excreted.

*Effects on the partition of the total urinary nitrogen.* The absolute excretion of urea nitrogen ran parallel with the urinary total nitrogen except in the eighth period. There was also the same general harmony in the relative elimination. The trend in urea elimination during the first five periods was practically the

reverse of that shown in the previous metabolism experiment. *Ammonia* nitrogen was eliminated in large absolute amounts in the first two dosage periods and in its maximal quantity during the post-subcutaneous injection period (fifth). During the last four periods the elimination was uniform and above the normal. The percentage figures in Table VIII agree with those for the absolute eliminations of ammonia. *Creatinin*, absolutely, was much the same quantitatively for each period, with the greatest elimination in the last period. Relatively the smallest excretion of creatinin occurred in the post-subcutaneous injection period (5th). There was relative period increase of creatinin to the third period, decrease to the minimum percentage value in the fifth period, and rise to the maximum percentage excretion at the end. *Allantoin*, both absolutely and relatively, was increased to the maximum values in the subcutaneous injection period (4th) and decreased slightly toward the end of the experiment. In all the periods except the second (*per os* administration) the quantity of allantoin was above normal. *Uric acid* was much the same in amount in each period. It was increased somewhat during the last two periods. Relatively it was least in amount in the post-subcutaneous injection period (5th) and highest in the last two periods. *Purin bases*, both absolutely and relatively, decreased in quantity sharply to the third period (*per os*), rose somewhat though still remaining at a subnormal point in the fifth (post-subcutaneous) and fell to very low amounts during the last two periods. The nitrogen in *undetermined products* fluctuated in absolute amounts to subnormal values in all but the third (ante-subcutaneous) and fifth (post-subcutaneous) periods. The absolute amounts of undetermined nitrogen in the last two periods were especially small. Relatively there was a fall to a low figure for undetermined nitrogen in the fourth (subcutaneous injection) period, a rise nearly to the normal percentage in the sixth (first intravenous injection) period, and a sharp fall to very low figures at the end.

*Elimination of magnesium per rectum.* The average daily eliminations of magnesium in the tenth and eleventh periods (p. 107), were the following: Tenth period, 6 days, 0.096 gram; eleventh period (after intravenous injection of 8.5 grams of  $MgSO_4$ ); 5 days, 0.103 gram; 7 days, 0.096 gram; 10 days, 0.096 gram.

# 112 Influence of Magnesium Sulfate on Metabolism

TABLE VII.

*Some of the daily records of the fourth experiment.*

*The second metabolism experiment.*

**FIRST PERIOD.** *Normal conditions. Days 1-7; February 18-24, 1908.*

Day No.	Body weight.	Dose of MgSO <sub>4</sub> .	URINE.						FECES.
			Volume.	Specific gravity.	Total nitrogen.	Urea nitrogen.	Creatinin nitrogen.	Ammonia nitrogen.	Dry weight.
	<i>kilos.</i>	<i>grams.</i>	<i>cc.</i>	<i>10xx.</i>	<i>grams.</i>	<i>grams.</i>	<i>gram.</i>	<i>gram.</i>	<i>grams.</i>
1	10.73		435	12				0.190	0
2	10.69		334	14	8.887	7.068	0.158	0.144	48.2
3	10.68		346	14				0.148	30.5
4	10.69		340	16	9.383	6.986	0.183	0.135	29.0
5	10.76		322	16				0.144	0
6	10.68		442	14				0.148	43.9
7	10.65		390*	13	14.664	8.737	0.340	0.122	29.0

\* By mistake 32 cc. of urine, which should have been added to the 8th day portion, were added to that of the 7th day, thereby changing the volume from 358 to 390 cc. and the weight of the dog from 10.68 to 10.65 kilos.

**SECOND PERIOD.** *Influence of magnesium sulfate administered per os. Days 8-14; February 25-March 2.*

8	10.73	3.001	310	17				0.202	0
9	10.78	3.998	312	19	9.371	6.846	0.183	0.252	43.0
10	10.76	5.002	330	17				0.279	53.5
11	10.68	5.004	390	16	10.007	6.834	0.208	0.278	40.2
12	10.75	5.000	300	18				0.257	0
13	10.73	5.002	346	20				0.293	24.9
14	10.73	5.001	345	18	14.737	10.382	0.328	0.288	39.5

**THIRD PERIOD.** *First intermediate period. Days 15-22. March 3-10.*

15	10.77		330	17				0.236	0
16	10.78		285	18	10.763	7.890	0.265	0.189	53.0
17	10.77		392	17				0.168	26.1
18	10.78		315	16	10.776	7.810	0.233	0.209	21.0
19	10.79		385	15				0.189	45.4
20	10.75		408	14	10.534	7.310	0.247	0.188	22.9
21	10.68		455	14				0.195	27.0
22	10.69		380	14	10.878	7.373	0.247	0.203	15.7

TABLE VII—CONTINUED.

FOURTH PERIOD. *Influence of magnesium sulfate injected subcutaneously.*  
*Days 23-26; March 11-14.*

Day No.	Body weight.	Dose of $MgSO_4$ .	URINE.						FECES.
			Volume.	Specific gravity.	Total nitrogen.	Urea nitrogen.	Creatinin nitrogen.	Ammonia nitrogen.	Dry weight.
	kilos.	grams.	cc.	10xx.	grams.	grams.	gram.	gram.	grams.
23	10.71	1.00	375	17				0.254	45.9
24	10.68	2.00	415	15	11.079	8.113	0.237	0.266	16.2
25	10.71	4.00	395	23				0.268	0
26	10.78	8.00	280	38	11.322	9.252	0.197	0.203	44.9

FIFTH PERIOD. *Second intermediate period. Days 27-31; March 15-19.*

27	10.71		450	23				0.395	33.5
28	10.64		400	15	15.817	11.914	0.263	0.444	15.0
29	10.53		433	21				0.636	32.9
30	10.54		389	17				0.348	42.5
31	10.52		410	16	21.375	18.006	0.330	0.380	27.1

SIXTH PERIOD. *Influence of magnesium sulfate injected intravenously.*  
*Days 32-36; March 20-24.*

32	10.47	8.00	460	23				0.184	0
33	10.48		270	21	11.698	8.852	0.239	0.258	63.5
34	10.48		325	19				0.236	26.0
35	10.53		362	15				0.219	0
36	10.49		340	15	15.444	10.701	0.345	0.204	54.4

## 114 Influence of Magnesium Sulfate on Metabolism

TABLE VII—CONTINUED.

SEVENTH PERIOD. *Third intermediate period. Days 37-45; March 25-April 2.*

Day No.	Body weight.	Dose of MgSO <sub>4</sub> .	URINE.						FECES.
			Volume.	Specific gravity.	Total nitrogen.	Urea nitrogen.	Creatinin nitrogen.	Ammonia nitrogen.	Dry weight.
	kilos.	grams.	cc.	10xx.	grams.	grams.	gram.	gram.	grams.
37	10.53		364	15				0.204	0
38	10.58		308	17				0.214	56.9
39	10.64		350	16				0.243	0
40	10.65		375	14				0.218	25.7
41	10.64		435	14				0.237	29.8
42	10.64		390	13				0.211	52.0
43	10.68		370	13				0.198	0
44	10.67		370	13				0.206	47.5
45	10.68		370	14	43.70			0.206	26.0

EIGHTH PERIOD. *New normal period. Days 46-51; April 3-8.*

46	10.70		395	13				0.185	0
47	10.74		360	14	9.233	7.636	0.253	0.200	37.5
48	10.70		465	13				0.225	53.0
49	10.71		405	14	10.049	8.743	0.249	0.208	12.8
50	10.75		310	13				0.202	24.9
51	10.78		330	15	9.128	7.738	0.241	0.206	40.7

NINTH PERIOD. *Second intravenous injection period. Days 52-57; April 9-14.*

52	10.57	7.00	665	17				0.141	27.0
53	10.59		355	14	10.968	9.589	0.290	0.272	27.9
54	10.65		365	14				0.223	24.5
55	10.71		368	15	10.788	9.039	0.273	0.237	25.5
56	10.76		345	14				0.202	52.5
57	10.78		375	14	10.477	9.035	0.245	0.233	21.5

TABLE VII—CONTINUED.

TENTH PERIOD. *Fourth intermediate period. Days 58-63; April 15-20.*

	58	59	60	61	62	63
Number of the day(period)	10.77	10.79	10.76	10.76	10.77	10.77
Body weight (kilos) . . . . .	403	345	416	410	400	390
Urine: Volume (cc.) . . . . .	14	14	13	13	13	14
Specific gravity (10xx) . . . . .	17.7	28.9	27.0	18.4	18.6	64.0
Feces: weight (grams) . . . . .						0.576*
Magnesium (gram) . . . . .						

\*Total for days 58-63.

ELEVENTH PERIOD. *Influence of an intravenous injection of magnesium sulfate on the content of magnesium in the feces.*  
*Days 64-73; April 21-30.*

	64	65	66	67	68	69	70	71	72	73
Number of day (period) . . . . .										
Body weight (kilos) . . . . .	10.59	10.64	10.69	10.72	10.75	10.77	10.78	10.77	10.78	10.78
Dose of MgSO <sub>4</sub> (grams) . . . . .	8.50									
Urine: volume (cc.) . . . . .	555	352	300	338	377	372	310	400	374	384
Sp. gr. (10xx) . . . . .	19	14	15	15	14	14	14	13	14	14
Feces: weight (grams) . . . . .	0	26.3	49.0	30.0	37.5	31.5	27.0	49.2	23.0	21.0
Magnesium (gram) . . . . .	0*	0.137	0.146	0.105	0.127		156†			0.289‡

\*No defecation.

†Days 69-70.

‡Days 71-73.





TABLE IX.

FOURTH EXPERIMENT. *Analytic totals and averages for nitrogen in each period.*

Period No. ... Days.....	I 7	II 7	III 8	IV 4	V 5	VI 5	VII 9	VIII 6	IX 6
	grams.	grams.	grams.	grams.	grams.	grams.	grams.	grams.	grams.
Food .....	42.04	42.04	49.72	24.86	31.08	31.08	55.94	36.57	36.57
Excreta...	36.42	37.20	46.57	24.35	39.71	29.75	47.67	31.07	35.04
Balance...	+5.62	+4.84	+3.15	+0.51	-8.63	+1.33	+8.27	+5.50	+1.53

TABLE X.

*Some of the daily records of the fifth experiment.*FIRST PERIOD. *Normal conditions. Days 1-9; March 30-April 8, 1908.*

Number of the day (period.).....	1	2	3	4	5	6	7	8	9
Body weight (kilos)...	15.83	15.77	15.88	15.74	15.73	15.88	15.94	15.95	15.96
Urine: Volume (cc.) ..	275	470	320	540	370	340	370	325	270
Specific gravity (10xx).....	23	24	21	20	24	22	22	20	25

SECOND PERIOD. *Intramuscular injection of magnesium sulfate.**Days 10-18; April 9-17.*

Number of the day (period.).....	10	11	12	13	14	15	16	17	18
Body weight (kilos)...	15.30	15.37	15.90	15.81	15.85	16.20	15.90	15.92	15.95
Dose of MgSO <sub>4</sub> (grams).....	12.00								
Urine volume: (cc.)...	860	220	0	455	350	0	680	495	308
Specific gravity (10xx).....	27	26		36	35		24	26	28

## 118 Influence of Magnesium Sulfate on Metabolism

### VII. FIFTH EXPERIMENT. DOES THE INTRAMUSCULAR INJECTION OF LARGE DOSES OF MAGNESIUM SULFATE INTO DOGS CAUSE THE DEVELOPMENT OF ABSCESSES?

Meltzer and Auer<sup>1</sup> found that in rabbits intramuscular injections of concentrated solutions of magnesium salts caused the development of abscesses. Subcutaneous injections of such solutions into rabbits did not cause abscesses. In dogs, however, Meltzer and Auer observed the reverse effects, i. e., abscesses after subcutaneous, but not after intramuscular, injections. In our experiments relatively large doses introduced subcutaneously always caused necrosis and sloughing in dogs. We took no aseptic precautions, however, in connection with the hypodermic treatment.

The outcome of the following experiment confirms the related observations by Meltzer and Auer:

A healthy, long haired, male dog weighing about 15.50 kilos was given a daily diet consisting of hashed lean beef, 225 grams; cracker meal, 60 grams; lard, 45 grams, bone ash, 30 grams; and 525 cc. of water.

FIRST PERIOD. *Normal conditions.* Days 1-9; March 30-April 8, 1908. The dog was kept on the above-mentioned diet for twenty-two days before the injection was made. Table X gives data pertaining to the last nine of the twenty-two days referred to.

SECOND PERIOD. *Intramuscular injection of magnesium sulfate.* Days 10-18; April 9-17. On the 23d day (first of the injection period), 12 grams of magnesium sulfate, dissolved in 30 cc. of water, were injected into the right hind leg. Immediately after the injection the dog was very restless. He squirmed about on the bottom of his cage, panted excessively for about half an hour, and then became drowsy and slept practically all evening, rising occasionally to urinate. On the next day he was still sleepy and was so lame that he would not rise of his own accord. During the night he urinated 860 cc. of very turbid urine; reaction to litmus, amphoteric. The dog's lameness continued for several days. There was some temporary swelling, but no abscess formation nor any sloughing. There was no diarrhea at any time. Recovery was prompt.<sup>2</sup>

### VIII. GENERAL COMPARISON AND DISCUSSION OF THE RESULTS.

A comparative examination of the data of all our experiments warrants the following condensed summary of the results of the treatment with magnesium sulfate.

<sup>1</sup>Meltzer and Auer: *Amer. Journ. of Physiol.*, xiv, p. 374, 1905.

<sup>2</sup>A second experiment under essentially the same conditions gave the same negative result.

**BODY WEIGHT.** On a rising scale of weight in the normal period of the first experiment, administration *per os* and some of those subcutaneously were accompanied by further increase in the body weight. The larger doses subcutaneously caused diuresis and decreased weight. There was increased weight in the post-subcutaneous period. On a rising scale of weight in the normal period of the special experiment referred to in the footnote on p. 104, administration subcutaneously caused diuresis and decreased weight. In the post-subcutaneous injection period body weight returned to and rose above normal. In the fourth experiment on a *falling* normal scale, administration *per os* and subcutaneously were associated with stationary or perhaps slightly increased weight, but there was decreased weight in the post-subcutaneous period with prompt recovery. Intravenous injection always caused immediate diuresis and decrease in body weight, with prompt recovery in a few days. In the second experiment the largest doses *per os* caused sufficient diarrhea to reduce body weight slightly. In the fifth experiment, intramuscular injection caused immediate diuresis and reduction of body weight. Recovery of weight was prompt.

**VOLUME OF THE URINE.** Disregarding normal variations in quantity, the volume of urine was generally decreased by administration *per os*, evidently because of the resultant diarrhea. In the second metabolism experiment there was apparently no effect on volume. Urinary volume was usually increased, at once or in a few days, by the larger subcutaneous doses. There was immediate diuresis after intravenous or intramuscular injections followed by very prompt compensatory reduction of volume. As a rule diuretic effects were soon completely balanced by corresponding retentions.

**SPECIFIC GRAVITY OF THE URINE.** Aside from its normal fluctuations specific gravity was relatively high after subcutaneous injections of large doses *per os*, obviously because of the rapid elimination (of the magnesium compound) that occurred usually without special diuretic effect. The marked diuresis that followed intramuscular or intravenous injections prevented special rise in specific gravity after such introductions. The specific gravity was usually high for a few days after the cessation of diuresis, evidently because of compensatory retention of water, as shown by the simultaneous elimination of relatively small urine volumes.

CONSISTENCY, AMOUNT, AND NITROGEN-CONTENT OF THE FECES. Administration of relatively large doses *per os* caused diarrhea. Subcutaneous intramuscular as well as intravenous injections failed to elicit diarrhea; on the contrary, such introductions seemed to make the feces drier and harder.<sup>1</sup>

In the first metabolism experiment the *dry* fecal matter increased per period to the maximum average daily elimination in the subcutaneous injection period (4th) and decreased to subnormal amounts in the post-subcutaneous injection and supplementary periods. In the second metabolism experiment the average daily quantity of dry matter in the fecal discharges was above the normal elimination in the second (administration *per os*) and all subsequent periods. The greatest eliminations in the second metabolism experiment appeared to occur as a rule after treatment with magnesium sulfate, whatever its channel of introduction.

The average daily content of nitrogen in the feces was ascertained in only one experiment—the second *metabolism* experiment. See the remarks in this connection on p. 109.

TOTAL NITROGEN BALANCE. The nitrogen balance was determined in the second metabolism experiment, but in no other. See Table IX; also p. 110.

TOTAL URINARY NITROGEN. In all periods except one<sup>2</sup> the total amount of recorded *urinary* nitrogen was above that of the normal period. In both metabolism experiments the maximal period excretion of urinary nitrogen occurred in the post-hypodermic injection period after cumulative increase to that period. Possibly the local disintegrating effects of the subcutaneous treatment was the prime factor in this result. Diuresis had evidently nothing to do with the outcome in this respect in the first metabolism experiment, but may have greatly influenced the result in the second metabolism experiment. Administration *per os* had no immediate effect if any on the elimination of total nitrogen. The high excretion values for total nitrogen, after subcutaneous injection, were not fully maintained in the subsequent intravenous injection periods, although as stated

<sup>1</sup> There were possible exceptions after subcutaneous injection in the fourth period of the first metabolism experiment (p. 89).

<sup>2</sup> First metabolism experiment, administration *per os* (second period).

above excretion of total nitrogen was still supernormal in each of them, and there was evidence of increased output of nitrogen as a consequence of the treatment intravenously.

**PARTITION OF THE TOTAL URINARY NITROGEN.** The comparative effects on the distribution of nitrogen among the leading urinary nitrogenous constituents is shown at a glance in Table XI, where the + sign means a value above that for the first normal period; a - sign shows a subnormal quantity; an = sign stands for no appreciable increase or decrease, as compared with the normal value and an asterisk indicates that no determination was made. A blank signifies that the experiment was not duplicated and that no comparative result was attained.

*Ammonia.* In a general way the tables show that ammonia was increased both absolutely and relatively in every period after the normal one.

*Urea.* The absolute amounts of urea were appreciably above normal in nearly every period although the relative excretion was subnormal after the period of *per os* administration in the third experiment.

*Creatinin* was essentially the same as urea in appearing in larger absolute amounts than the normal in practically all post-normal periods. Its *relative* excretion showed somewhat less uniformity than urea's.

*Allantoin* was like urea in passing into the urine in supernormal amounts in nearly all the post-normal periods. Relatively its excretion tendency was also similar to urea's.

*Uric acid* was increased both absolutely and relatively in a bare majority of the post-normal periods.

*Purin bases.* The nitrogen of purin bases exhibited diametrically opposite tendencies in the two experiments, both absolutely and relatively. Purin base nitrogen was in the main increased absolutely and relatively in Experiment III, but decreased in both respects in Experiment VI.

*Undetermined.* The remarks made about the nitrogen of purin bases apply to that of the undetermined products.

TABLE XI.

Summary of comparative data on the absolute and relative partition of total urinary nitrogen in the first and second metabolism experiments (Experiments III and VI).

## A Absolute amounts.

No.	Period. Condition.	URINARY NITROGEN.							
		Total. III VI	Urea. III VI	Ammonia III VI	Creatinin. III VI	Allantoin. III VI	Uric acid. III VI	Purin base. III VI	Undeter- mined. III VI
II	MgSO <sub>4</sub> per os.....	+	+	+	+	=	-	+	-
III	First intermediate.....	+	+	+	+	+	-	-	+
IV	MgSO <sub>4</sub> subcutaneously.	+	-	+	+	+	+	+	-
V	Second intermediate....	+	+	+	=	+	+	+	-
VI	MgSO <sub>4</sub> intravenously..	+	+	+	+	+	-	-	+
VII	Third intermediate.....	+	*	+	*	*	*	*	*
VIII	New normal.....	+	+	+	+	+	+	-	-
IX	MgSO <sub>4</sub> intravenously..	+	+	+	+	+	+	-	-

## B Relative values.

II	MgSO <sub>4</sub> per os.....	+	+	+	+	+	+	+	-
III	First intermediate.....	+	+	+	+	+	+	+	-
IV	MgSO <sub>4</sub> subcutaneously.	+	+	+	+	+	+	+	-
V	Second intermediate....	+	+	+	+	+	+	+	-
VI	MgSO <sub>4</sub> intravenously..	+	+	+	+	+	+	+	-
VII	Third intermediate.....	+	*	+	*	*	*	*	*
VIII	New normal.....	+	+	+	+	+	+	+	-
IX	MgSO <sub>4</sub> intravenously..	+	+	+	+	+	+	+	-

GENERAL COMPARISONS. Considering the data pertaining to the partition of nitrogen from the standpoint of direct agreement in the two experiments it is obvious that the increased elimination of ammonia in all the post-normal periods is the most striking and perfect concordance. That this increase was largely due to the combination of ammonia with magnesium in the form of ammonio-magnesium phosphate appears to be certain. The increase indicated by our figures in the third experiment was doubtless below the full increase that would have been exhibited by a perfect method of determining ammonia nitrogen when in the form of "triple phosphate." In the sixth experiment, however, possibly all the ammonia was recovered by the modified method employed.

The nitrogen of urea, creatinin and allantoin, taken collectively appeared in increased amounts, both absolutely and relatively in a majority of the post-normal periods.

The results for nitrogen of uric acid, purin bases and undetermined products were too discordant to permit of any such deduction one way or the other.

#### IX. SUMMARY OF GENERAL CONCLUSIONS.

Disregarding matters of detail that have been referred to in connection with the discussion of the data of each experiment, the following conclusions sum up the main results of the research:

In these experiments, in which relatively large doses of magnesium sulfate were given to dogs, abscesses and sloughing followed subcutaneous injections, but were not caused by intramuscular or intravenous injections nor by administration *per os*. Very large doses of magnesium sulfate could repeatedly be injected intravenously without causing death, when care was taken to conduct the process slowly.

Fluctuations in the weight of the animals, as well as in the volume and specific gravity of their urines, resulted chiefly from diuretic or diarrheal influences and the consequent compensatory tendencies.

Administration *per os* caused diarrhea. Bone ash in the food appeared to exert only a mechanical diminution of such diarrheal tendencies. Injections under the skin or into a muscle or into

the circulation failed to elicit any evidence of diarrhea, except in one doubtful case after subcutaneous application. On the contrary, such injections appeared to make the feces drier and harder than ordinarily, and the urine volumes greater.

If there was any effect on the quantitative elimination of solid matter in the feces, it was not more than a slight increase. The same may be said of the contents of magnesium and nitrogen in the feces.

In a general way elimination of nitrogen in the urine was increased after the normal periods, but the increase was not sufficient to warrant the conclusion that it was a direct effect of the dosage. The absolute increase of urinary nitrogen observed was registered chiefly in the form of urea, although the relative excretion of the latter was below normal in Experiment III.

The most striking and consistent effect on the partition of the urinary nitrogen was the continued absolute as well as relative increase of ammonia elimination throughout the whole of the dosage part of each metabolism experiment, in spite of the fact that the Folin method does not permit of complete recovery of ammonia from crystallized ammonio-magnesium phosphate. The increased elimination of ammonia nitrogen may be attributed, in large part at least, to special formation and elimination of ammonio-magnesium phosphate under the prevalent conditions. The observations pertaining to the remaining nitrogenous constituents of the urine have been summarized on p. 121.

It is especially noteworthy that recovery from dosage with magnesium sulfate, however profound the immediate effect of such treatment may have been, was always prompt and apparently complete so far as general observation and our data indicated. That magnesium sulfate exercises surprisingly little effect on nitrogen metabolism under the conditions of these experiments has also been shown by the results.

In conclusion it gives me great pleasure to acknowledge my sincere gratitude to Prof. William J. Gies, at whose suggestion these experiments were begun and to whose constant advice I owe their completion.



# A NOTE ON THE CHEMISTRY OF THE MUSCLE AND LIVER OF REPTILES.

By JOHN F. LYMAN.

(From the Sheffield Laboratory of Physiological Chemistry, Yale University.)

(Received for publication, May 2, 1908.)

The paucity of data regarding the chemistry of the lower vertebrates affords the occasion for recording a few observations made at the suggestion of Prof. Lafayette B. Mendel, on the muscles and liver of pythons. Aside from the studies of Krukenberg<sup>1</sup> we know of no investigations on these tissues in reptiles. This author states that he found creatin and hypoxanthin in the muscle extract of *Python sabbæ*, *Python molurus*, and an alligator. Through the kindness of Dr. L. J. Cole, we obtained 4 kilograms of fresh muscle and the livers from two specimens of *Python reticularis* (?) which had just died. The water extracts of the muscles were precipitated with alcohol, the filtrates concentrated under diminished pressure, treated with lead acetate, and the lead-free solution allowed to concentrate until a crop of crystals was obtained. At subsequent stages in the examination of the extract more crystals were separated, making a total of 8.2 grams which were identified as *creatin*.<sup>2</sup>

	Found:	Calculated:
H <sub>2</sub> O in C <sub>4</sub> H <sub>9</sub> N <sub>3</sub> O <sub>2</sub> .H <sub>2</sub> O...	12.15 per cent	12.08 per cent
N in C <sub>4</sub> H <sub>9</sub> N <sub>3</sub> O <sub>2</sub> .....	32.0 " "	32.15 " "

The filtrate from the bulk of the creatin was concentrated to a syrup, which was repeatedly extracted with hot alcohol. After removal of the alcohol the solution was acidified with sulphuric acid and extracted with ether in the Kutscher-Steudel apparatus.

<sup>1</sup> Krukenberg: *Vergleichend-physiologische Studien*, ii Reihe, ii Abtheilung, p. 81, 1882.

<sup>2</sup> Professor Mendel has separated creatin from the muscles of the turtle *Chelone midas*.

## 126 Chemistry of Muscle and Liver of Reptiles

From the extract 2.23 grams of zinc paralactate were prepared in the usual manner and identified by analysis:

	Found:	Calculated for (C <sub>3</sub> H <sub>5</sub> O <sub>3</sub> ) <sub>2</sub> Zn.2H <sub>2</sub> O:
H <sub>2</sub> O.....	13.0 per cent	12.9 per cent
Zn.....	24.3 " "	23.8 " "

The zinc salt was lævorotatory. It is interesting to note in this connection that fermentation lactic acid has been found in the muscles of invertebrates by Henze<sup>1</sup> in *Octopus*, and by Mendel and Bradley<sup>2</sup> in *Sycotypus*.

After removal of sulphuric acid with barium carbonate the purins were separated from the residual extract by precipitation with an ammoniacal solution of silver chloride. As in the case of the skeletal muscles of the higher vertebrates, nonstriated vertebrate muscle,<sup>3</sup> and embryonic vertebrate muscle,<sup>4</sup> *hypoxanthin* was found to be the conspicuous purin derivative. Guanine, adenine, and xanthine could not be isolated from the material available. The hypoxanthine (0.33 gram) was identified as the nitrate:

	Found:	Calculated for C <sub>5</sub> H <sub>4</sub> N <sub>4</sub> O. HNO <sub>3</sub> . H <sub>2</sub> O:
N.....	32.4 per cent	32.3 per cent

The fresh livers, weighing 440 grams, were comminuted and boiled 4 hours with 5 per cent sulphuric acid. From the filtrates the purin derivatives were separated by the copper sulphate-sodium bisulphite method according to the direction of Schittenhelm.<sup>5</sup> The products isolated were:

*Uric acid*, 0.092 gram, recrystallized according to Horbaczewski's method:

<sup>1</sup> Henze: *Zeitschrift für physiologische Chemie*, xliii, p. 477, 1904.

<sup>2</sup> Mendel and Bradley: *American Journal of Physiology*, xvii, p. 169, 1906.

<sup>3</sup> Saiki: *This Journal*, iv, p. 483, 1908.

<sup>4</sup> Mendel and Leavenworth: *American Journal of Physiology*, xxi, p. 102, 1908.

<sup>5</sup> Schittenhelm: *Deutsches Archiv für klinische Medizin*, lxxxi, p. 450, 1904.

	Found:	Calculated for $C_5H_4N_4O_3$ :
N.....	33.99 per cent	33.33 per cent

*Guanin*, 0.39 gram, which was analyzed as the hydrochloride:

	Found:	Calculated:
$H_2O$ in $C_5H_5N_5O.HCl.2H_2O$ ...	16.8 per cent	16.1 per cent
N in $C_5H_5N_5O.HCl$ .....	37.2 " "	37.4 " "

*Adenin*, separated as the picrate, 0.62 gram being obtained. It was still impure after recrystallization, melting at about  $265^{\circ}$  C. instead of  $281^{\circ}$ . Hypoxanthin and xanthin could not be separated in amount sufficient for identification. From the preceding observations it is apparent that the same purins are present in the nucleoprotein complexes of these species as in the higher vertebrates. The presence of uric acid is associated with the well known synthetic capacity of reptiles in relation to the end products of nitrogenous metabolism.



## THE CHEMISTRY OF THE LIVER IN CHLOROFORM NECROSIS (DELAYED CHLOROFORM POISONING).

By H. GIDEON WELLS.

(From the Pathological Laboratory of the University of Chicago.)

(Received for publication, June 23, 1908.)

Recently I reported the results of a complete analysis of a liver exhibiting the typical conditions of acute yellow atrophy,<sup>1</sup> and while engaged in this analysis had the good fortune, through the kindness of Dr. F. S. Tufts, to perform an autopsy upon the body of a young man who had died a few hours before from the condition of acute necrosis of the liver that occasionally follows the administration of chloroform. This liver has now been analyzed in the same manner as was the acute yellow atrophy liver, and certain steps have been duplicated with two normal livers for comparison, all four livers having been obtained from robust young men in the third decade of life. Some of the final figures of all four analyses were included in the paper upon acute yellow atrophy, but in the following report will be considered particularly the chemistry of the liver in chloroform necrosis.

Delayed chloroform poisoning is an extremely serious, often fatal sequence of the use of chloroform as an anesthetic, which has only recently become generally recognized. Attention was called to it in this country particularly by the articles of Ballin and of Bevan and Favill,<sup>2</sup> and in England by Guthrie,<sup>3</sup> while the chief recent article in German literature is that of Guleke.<sup>4</sup> It is not my purpose here to go into the literature, or to discuss the clinical and anatomical features of the case, which has been done more extensively in another place.<sup>5</sup> Briefly, the features of the effects

<sup>1</sup> *Journ. of Exper. Med.*, ix, p. 627, 1907.

<sup>2</sup> *Journ. Amer. Med. Assoc.*, xix, September 2, 1905.

<sup>3</sup> *Lancet*, July 4, 1903.

<sup>4</sup> *Arch. j. klin. Chir.*, lxxxiii, p. 602, 1907.

<sup>5</sup> *Archives of Internal Medicine*, i, p. 589, 1908.

of delayed chloroform poisoning are as follows: After an operation, which may involve any part of the body but which is most frequently abdominal, and in which chloroform has been used as the anesthetic,<sup>1</sup> the patient begins as if upon the road to an uneventful recovery; but after a period of from 12 to 72 hours (generally about 24 to 36 hours), symptoms of restlessness, fear and delirium appear, passing into coma which terminates after two to four days, as a rule, in death. The persons are almost always young persons, never past middle life, and the cases reported in the literature seem to fall into two groups. In one, which is seen chiefly in children, the manifestations are similar to those of acid intoxication, with the so-called acetone odor of the breath and the presence of acetone and diacetic acid in the urine, more or less cyanosis, and sometimes distinct air hunger; anatomically in these cases there is usually found a well marked fatty degeneration of the kidneys, myocardium and liver, the changes in the latter being described as chiefly in the periphery of the lobules. The other type of poisoning has been observed in young adults, and differs in that with the onset of delirium jaundice appears, and usually becomes profound, cutaneous hemorrhages often develop, there is more or less tenderness in the region of the liver, the area of liver dulness decreases, leucin and tyrosin may be found in the urine, and the clinical picture is similar to that of acute yellow atrophy; at autopsy the liver is found decreased in size, friable, yellow, extremely degenerated, with necrosis of by far the majority of the liver cells, and there is more or less necrosis and fatty degeneration of the kidneys.

The case that I have studied is of the second type, in which the hepatic changes are for the most part similar to those of acute yellow atrophy, and may be described briefly as follows: A vigorous young man, 28 years of age, was operated upon for gallstones, chloroform being given in a rather liberal manner. The operation, which amounted merely to an exploratory laparotomy, was of short duration, and for the first 48 hours after the patient was in good condition; then restlessness began, rapidly passing into delirium and coma; jaundice appeared and became pronounced,

<sup>1</sup> There are, perhaps, a few instances in which a similar effect has been produced by ether.

and death occurred 100 hours after the operation. At the autopsy, performed twelve hours after death, the chief changes were found in the liver, which had been reduced in size so that it weighed but 1050 grams, this being rather under than over two-thirds the normal weight for a man of this size and age. It had a wrinkled capsule, and was strikingly shrunken, flabby, soft and friable; it appeared as if badly decomposed, except that it had a sweetish odor, and it was of a yellow color on the cut surface. Microscopically the cells in the center of each lobule were found necrosed, greatly decreased in size and number, granular, containing many small droplets, and causing an extremely disorganized appearance of at least two-thirds to three-fourths of each lobule; only the cells at the very periphery of each lobule still retain the power of taking the nuclear stains, and even they are very granular and contain numerous large droplets of fat. These changes are quite similar to those seen in the early stages of acute yellow atrophy, differing chiefly in the considerable amount of fatty change that is present.

#### CHEMICAL ANALYSIS.

Immediately after the autopsy the liver was cut into thin slices, and 850 grams (which constituted 81 per cent of the entire liver) was placed in a large volume of 95 per cent alcohol. After hardening was completed the tissue was ground up in a meat chopper and extracted repeatedly with fresh quantities of boiling alcohol under a reflux condenser, the alcoholic extracts being filtered off and united. Twice the residue was dried and ground to a powder in a mill during the alcohol extraction, and after this it was extracted in a Soxhlet apparatus with absolute ether until nothing more could be removed. The ether and alcohol extracts were united and evaporated to dryness under vacuum at a temperature not exceeding 40°, the residue obtained weighing 127.5 grams, or 15 per cent of the entire fresh weight of liver taken. A sample of the fresh liver tissue was also weighed and dried, and the water content was found to be 72.4, leaving 27.6 per cent of solids. The liver residue left after this extraction was then extracted in a shaking machine repeatedly with fresh quantities of water, then with water at 50° to 60°, these extracts

being united and evaporated to dryness at 40° to 45° *in vacuo*; the weight of the dried extract was 5.03 grams. The residue of liver tissue was then extracted with boiling water, changed frequently, until the extracts were practically colorless. These three distinct extracts (alcohol and ether, cold and warm water, hot water) and the insoluble residue of coagulated liver tissue were now available for separate analysis.

The hot water extract was concentrated to 100 cc., and four volumes of absolute alcohol added; the precipitate was filtered off, washed in 95 per cent alcohol, redissolved in hot water and reprecipitated with alcohol. On dissolving this precipitate again it was found to give a good Millon's reaction, a bluish biuret reaction, but not the Hopkins-Cole reaction for tryptophan. On cooling it gelatinized, and when dialyzed for three days under toluol in a celloidin sac the diffusate contained no appreciable amount of solids and did not give the biuret reaction, indicating the absence of proteoses and peptones. Therefore the precipitate was pure, or nearly pure, gelatin. Determination of nitrogen in an aliquot part of the solution indicated a total of 0.302 gram nitrogen, which corresponds to 1.68 gram of gelatin (assuming that gelatin contains 18 per cent of nitrogen); this amounts to but 0.72 per cent of the total solids of the liver.

Evaporation of the alcoholic filtrate from the gelatin precipitation yielded 3.81 grams. of solids, which was added to the 5.03 grams of residue from the cold and warm water extracts; determination of the nitrogen in an aliquot part of this mixture indicated the presence of 0.894 gram of nitrogen, or 10 per cent of the total solids in these watery extracts. This solution gave good biuret and Millon reactions, and a faint reaction for tryptophan. On addition of several volumes of alcohol to the watery solution a granular precipitate was obtained, which was found to consist chiefly of arsenic from the embalming fluid.<sup>1</sup> The filtrate gave a strong biuret reaction, and upon concentration to 50 cc. and addition of five volumes of absolute alcohol there was obtained a typical hygroscopic proteose-peptone mixture, which weighed

<sup>1</sup> An attempt at embalming had been made, but fortunately for my purposes this was very unsuccessful; the liver was not at all hardened except a small part of the surface where embalming fluid injected into the peritoneal cavity had come in contact with it.



three grams when dry. This proteose-peptone mixture was then dissolved in 50 cc. of water and precipitated fractionally with ammonium sulphate according to the method of Pick, with the following result:

*First fraction* (half saturation). A brown gummy mass, weighing about 0.5 gram. This gave a slightly purplish biuret reaction, and was probably a mixture of gelatin and proteoses.

*Second fraction* (two-thirds saturation). About 0.2 gram more of brownish gummy precipitate, which gave a good pink biuret reaction and a good tryptophan reaction.

*Third fraction* (saturation). Only a very slight precipitate, giving a good biuret reaction and fair tryptophan reaction.

*Fourth fraction* (filtrate). Gave only a faint biuret reaction, therefore probably only a small amount of peptone present.

COMPOSITION OF THE INSOLUBLE COAGULATED RESIDUE. The residue of insoluble liver substance left after all the extractions had been completed, was dried to a constant weight and analyzed. The total nitrogen was determined by the Kjeldahl method in a sample, and found to amount to 14.48 per cent of the entire weight, indicating that the substance is nearly pure protein. The distribution of this nitrogen in the form of monamino, diamino and amid nitrogen was determined in two samples according to the Hausmann method as used by Osborne and Harris<sup>1</sup> with the following results:

	I.	II.	Average.	Per cent total nitrogen.
Amid nitrogen.....	0.52	0.58	0.55	3.9
Humus nitrogen.....	0.82	0.80	0.81	5.7
Diamino nitrogen*.....	4.25	4.17	4.21	30.0
Monamino nitrogen†.....	8.45	8.45	8.45	60.3
Totals.....	14.04	14.00	14.02	

\* This fraction also contains part of the purins.

† The monoamino nitrogen was determined directly in an aliquot part of the filtrate from the phosphotungstic acid precipitate, and not by difference as is usually done. On this account the total nitrogen obtained, 14.02 per cent is a trifle less than all the nitrogen present, 14.48, but this statement of results is probably more nearly accurate than when the determination is made by difference.

<sup>1</sup> Journ. Amer. Chem. Soc., xxv, p. 323, 1903.

Other portions of this liver residue were also analyzed for sulphur, iron and phosphorus, with the following percentage results:

	I.	II.	Average.
Sulphur.....	0.80	0.78	0.79
Phosphorus*.....	0.85	0.83	0.90
Phosphorus.....	0.98	0.93	
Iron.....	0.42	0.57	0.50
Ash.....	0.93	0.98	0.95

\* On account of the unexpectedly high figure for phosphorus in this analysis, in which the combustion was made by fusing with sodium carbonate and sodium nitrate, another pair of determinations was made by Neumann's method, which gave the slightly higher figures seen in the second row.

**FATS AND LIPOIDS.** The extracts with alcohol and ether, which had been evaporated to dryness *in vacuo*, weighed 127.5 grams, and this material was in turn thoroughly extracted with ether, which dissolved out 75 grams, presumably fats and lipoids, this constituting 8.8 per cent of the total fresh weight or 32 per cent of the solids of the liver. Therefore 18.8 per cent of the entire weight of the fresh liver was proteins and extractives, 8.8 per cent fats and lipoids, and 72.4 per cent water. The ether extract was evaporated to dryness, dissolved in absolute alcohol and made up to 250 cc., of which two 20 cc. samples were taken for cholesterin determinations and two 10 cc. samples for lecithin.

*Cholesterin* was determined according to the method of Ritter,<sup>1</sup> in which the fats are saponified with sodium alcoholate, dried out in a large volume of salt, and extracted thoroughly with ether which dissolves out the cholesterin but not the soaps. Traces of sodium alcoholate, soaps, and glycerin escaping into the ether extract are removed by shaking out with water, and the cholesterin weighed directly after evaporating off the ether. The amount found in the samples taken (0.353 gram in  $\frac{2}{25}$  of the extract) corresponds to a total of 4.4 grams in the 850 grams of fresh liver substance analyzed, or 5.4 grams in the entire liver; this indicates that cholesterin constituted 0.52 per cent of the fresh weight; 1.9 per cent of the total dry weight, 2.9 per cent of the fat-free dry substance, or 5.9 per cent of the ether-soluble material.

<sup>1</sup> *Zeitschr. f. physiol. Chem.*, xxxiv, p. 430, 1902.

*Lecithin* was determined by the method recommended by Koch and Woods<sup>1</sup> in which the lecithin is precipitated from an aqueous emulsion with acid chloroform, combusted by Neumann's method, and the phosphorus determined as magnesium pyrophosphate. The amount of phosphorus present, 0.0212 gram, in the samples analyzed, corresponds to 0.558 gram of lecithin in the sample, or 12.95 grams in the extract from 850 grams of liver, representing a total of 16 grams of lecithin in the entire liver. This indicates that lecithin constituted 1.5 per cent of the fresh weight, 5.5 per cent of the total solids, 8.1 per cent of the fat-free solids, and 17.3 per cent of the ether-soluble material.

AMINO-ACIDS AND PURINS. These were sought in the part of the original alcohol extract that was left after ether extraction, and in the non-protein portions of the watery extracts. These were united, dissolved in water, and made up to 1000 cc., and the nitrogen in a 10 cc. sample determined by Kjeldahl's method, 46.41 mg. of nitrogen was found in the sample, corresponding to 4.641 grams of nitrogen in the entire extract, the total weight of which was 45 grams. This material gave a strong Millon reaction, but only a very faint biuret reaction. It was examined for amino acids and purins, following with slight modifications the method used by Schumm<sup>2</sup> in his study of autolysis in leukemic spleens. As Schumm has given his procedure in detail it is unnecessary to give here the details of the analysis. Briefly, the diamino acids and purins were separated from the monamino acids by precipitating in 5 per cent sulphuric acid solution with phosphotungstic acid. From this precipitate, which contained 1.386 gram of nitrogen, were isolated the following substances:

I. *Free purins*. The total quantity obtained contained 0.0974 gram nitrogen, and from it was isolated 0.16 gram of hypoxanthin silver nitrate, and a smaller quantity of xanthin silver. Adenin and guanin could not be found.

II. *Diamino acids*. After removal of the purins from the phosphotungstic acid precipitate, the diamino acids were sought according to the method of Kossel and Kutscher. In the histidin fraction was found 91 mg. of nitrogen, which corresponds to

<sup>1</sup> This *Journal*, i, p. 203, 1906.

<sup>2</sup> *Beitr. z. chem. Physiol. u. Pathol.*, vii, p. 175, 1906.

0.34 gram of histidin. The arginin fraction contained but 32.2 mg. of nitrogen, and yielded a small amount of long rhombic crystals, but the amount was so small that it is impossible to tell whether this was arginin or not.

The fraction that should have contained the lysin contained 0.404 gram of nitrogen, but from it no lysin picrate could be obtained; instead there was a gummy mass, which gave a strong biuret and good Millon and tryptophan reactions. This was soluble in strong alcohol, and only a small part was precipitated by saturating the solution with ammonium sulphate; the filtrate from this precipitate gave strong biuret and Millon reactions, and a faint tryptophan reaction, and therefore was either peptone or polypeptid, most probably chiefly the latter.

III. *Monamino acids.* After removal of the phosphotungstic and sulphuric acids from the filtrate from the diamino acid precipitate, the filtrate was concentrated and by fractional crystallization was obtained 2.70 grams of what seemed to be chiefly a mixture of leucin and tyrosin. By extraction with glacial acetic acid 0.26 gram of pure tyrosin was obtained from the insoluble residue, which was identified by its typical cottony appearance when crystallizing out after purification, its insolubility in glacial acetic acid, and its intense Millon reaction.

By careful recrystallization of the filtrate from the tyrosin after removal of the acetic acid and decolorization with animal charcoal, leucin was obtained to the amount of 1.5 gram. This was converted into a copper salt by boiling with fresh copper oxide, and the typical bluish-white copper salt of leucin was readily obtained in pure condition. After drying this at  $112^{\circ}$  a nitrogen determination was made, and 8.77 per cent of nitrogen was obtained, the theory for the copper salt of leucin being 8.67 per cent of nitrogen. Copper was determined, and found to be 19.9 per cent, the theory for the copper salt of leucin being 19.6 per cent.

Upon saturating with hydrochloric acid gas the concentrated filtrate from the leucin-tyrosin crystallization, a considerable amount of crystalline material came out, but on further examination this was found to be entirely inorganic, and not glutamic acid hydrochloride. The filtrate from this was then esterified three times according to the method of Emil Fischer, and about

10.5 grams of raw esters was obtained. This was fractionated in the usual manner except that only the water pump was used, the pressure throughout the distillation being 9 to 12 mm., and the following fractions were obtained.

				Grams.
Fraction	I.	Temp. up to 60°; weight of esters.....		4.0
"	II.	" 60°-100°; " " .....		1.9
"	III.	" 100°-188°; " " .....		1.3
"	IV.	Residue " " .....		2.5

The first three fractions were hydrolyzed by boiling in water eight hours with inverted condenser, and then evaporated to dryness *in vacuo* over sulphuric acid at 45°. Fraction I yielded 0.58 gram of a crystalline white substance having a sweet taste and melting at 240°; probably chiefly glyocoll. Fraction II yielded 0.99 gram of a substance similar to fraction I, except that it had a mixed sweet and bitter taste and contained a few waxy crystals resembling leucin; it melted at 250° and was presumably a mixture of glyocoll with small amounts of higher amino acids. Fraction III yielded 0.81 gram of a brownish, semi-solid substance, smelling like prolin and slowly forming a small amount of crystalline substance.

Fraction IV was shaken out with ether and water until the ether-soluble portion was removed. This ether extract was hydrolyzed by evaporating with concentrated hydrochloric acid, but only a very small amount of crystalline material, mixed with a brownish amorphous substance, was obtained, insufficient for purification and identification as phenylalanin which would be found in this fraction if present. The watery extract was hydrolyzed by boiling with barium hydroxide, and the barium salts allowed to crystallize out, but after removing the barium from the crystalline material no aspartic acid or other crystalline organic substance could be found. The filtrate from the crystalline barium salts was freed from barium, concentrated to a few cubic centimeters, and saturated with hydrochloric acid gas; after standing on ice over night without crystallization the solution was inoculated with a crystal of glutamic acid hydrochloride and a considerable amount of typical crystals of this salt came out. This salt was separated by filtration through asbestos in a Gooch crucible, and weighed 0.58 gram. It was recrystallized,

and a nitrogen determination made, 7.74 per cent of nitrogen being found, agreeing well with the theory for the hydrochloride of glutamic acid, which calls for 7.63 per cent of nitrogen.

The chlorine was removed from the filtrate from the glutamic acid with lead oxide, and after removal of the lead with hydrogen sulphide a very small amount of slightly crystalline greenish material was obtained. This was redissolved and a copper salt was made, which was of a greenish yellow color and manifestly impure; therefore it was impossible to determine whether aspartic or any other amino acid was present in this fraction.

Fractions I and II were united, dissolved in 10 cc. of hot water, and on addition of three volumes of hot absolute alcohol there was obtained no precipitate, and none appeared on cooling, indicating the absence of any appreciable amount of leucin or alanin. Therefore the solution was evaporated to dryness, dissolved in a minimum amount of hot absolute alcohol, saturated with dry hydrochloric acid gas and cooled. After inoculating with crystals of the hydrochloride of glycocoll ethyl ester a small amount of crystals of this type appeared. The yield being unsatisfactory the chlorine was removed with lead oxide, and a copper salt was made of the amino acid in the filtrate. On analysis of the first fraction of this salt obtained on crystallization 13.58 per cent of nitrogen and 30.2 per cent of copper were obtained, the theory for the copper salt of glycocoll being nitrogen, 13.40 per cent, copper 30.8 per cent. Therefore, most if not all of the first two samples, amounting to 1.57 gram, is glycocoll.

The third fraction, which resembled prolin, was made into a copper salt, and the salt treated with absolute alcohol which dissolved out a greenish, noncrystalline material, leaving the crystals of the copper salt. This was analyzed for nitrogen, and 8.0 per cent found. The theory for the copper salt of prolin is 9.62 per cent, for aspartic acid 7.22 per cent, and for leucin 8.67 per cent. Possibly the salt obtained was an impure mixture, but the amount available for analysis was too small to permit of duplicate analyses or for copper determinations; therefore the presence or absence of prolin cannot be determined, but from the appearance and odor of the material it is probable that prolin was present.

## DISCUSSION OF RESULTS.

This analysis has most interest when compared with the results obtained by the analysis of two normal human livers and a liver from a case of typical "idiopathic" acute yellow atrophy, which has been previously published,<sup>1</sup> all four livers being from young men of about the same age. In the acute yellow atrophy liver the most interesting result was the isolation of a comparatively large number of amino acids in sufficient purity for their identification; in the chloroform necrosis a somewhat smaller number and smaller amounts were isolated, as shown by the following table:

	Acute atrophy	Chloroform necrosis
Histidin.....	0.64	0.34
Arginin.....		?
Lysin.....	1.04	?
Tyrosin.....	0.70	0.26
Leucin.....	4.16	1.50
Glycocoll.....	0.20	1.57
Alanin.....	0.30	
Prolin.....	0.35	present ?
Glutamic acid.....	1.00	0.58
Aspartic acid.....	0.28	
Phenylalanin.....		?
Total.....	8.67	4.25

With these results may be incorporated the results obtained by A. E. Taylor, who has also examined for amino acids the extracts from a case of acute yellow atrophy<sup>2</sup> and one of chloroform necrosis.<sup>3</sup> From the acute yellow atrophy liver he isolated 0.35 gram of leucin and 0.612 gram of aspartic acid; from the chloroform necrosis 2.3 gram of arginin nitrate, 2.2 grams of tyrosin, and 4.0 grams of leucin.

These figures of themselves indicate nothing as to the actual quantity of free amino acids present, on account of the inadequacy of the analytic methods that are available for their isolation; in each case the amino acids recovered account for only a

<sup>1</sup> *Journ. of Exper. Med.*, ix, p. 627, 1907.

<sup>2</sup> *Journ. of Med. Research*, viii, p. 424, 1902.

<sup>3</sup> *Univ. of Calif. Publ. (Pathol.)*, i, 43, 1904.

small fraction of the nitrogen present in the solutions containing them, and less than one-fourth of the total weight of the esters obtained from the chloroform necrosis liver could be recovered as amino acids. Nevertheless it is of interest to find that so many of these constituents of the protein molecule can be found free in degenerated livers, even if only in small amounts. These two analyses furnish the only instances that I can find in the literature of the isolation of free glutamic acid and free prolin from either human or animal tissues or excretions; the identity of the glutamic acid was completely established in both cases, but the prolin, although almost certainly present in the extracts from the chloroform necrosis liver, could not be isolated in sufficient amount for analysis and identification.

It might be expected that more free amino acids would be found in the chloroform necrosis liver, in which the reduction in size of the autolyzing liver took place in a few days, than in the acute yellow atrophy liver in which the process was of some six weeks' duration. The fact that a smaller quantity of amino acids was isolated from the chloroform necrosis liver may only depend upon less success with the analysis, but it may mean that there actually was a larger amount of free amino acids in the acute yellow atrophy liver. If the latter explanation is correct then we should be obliged to consider it as added evidence that in acute yellow atrophy the free amino acids found in the liver and secretions are not derived solely, or even chiefly, from the autolyzing liver cells. Neuberg and Richter<sup>1</sup> found larger amounts of free amino acids in the blood in acute yellow atrophy than could be accounted for by the destruction of liver tissue going on at the time, and concluded that there must be some other source for them, possibly the intestine. The rather large amount of amino acids isolated from the liver in the case of acute yellow atrophy mentioned above is in favor of the same idea, and the smaller amount present in the more rapidly digesting liver with chloroform necrosis might be looked upon as of similar significance, were not the value of quantitative results obtained with such materials and methods so very questionable.

Proteoses and peptones were also present, and apparently much more abundantly in the chloroform necrosis liver, in the

<sup>1</sup> *Deutsch. med. Woch.*, xxx, p. 499, 1904.



extract from which were probably also considerable amounts of substances related to the polypeptids. The relative abundance of these substances intermediate between proteins and amino acids in the chloroform necrosis liver is presumably dependent upon the fact that here the autolysis was less advanced than in the acute yellow atrophy liver.

In both livers the presence of free xanthin and free hypoxanthin was established, and in about the same amount in each. Free guanin and adenin were absent from both livers, presumably because they are so readily converted into xanthin and hypoxanthin by the hepatic enzymes.

The composition of the coagulated and insoluble proteins of the liver left after thorough extraction with alcohol, ether, cold and hot water, is found to be quite the same in chloroform necrosis as in normal livers, as shown by the following table giving the results of analysis by Hausmann's method:

	Acute atrophy.	Normal (anemic).	Normal (congested).	Chloroform necrosis.
Amid nitrogen.....	5.5	3.7	4.8	3.9
Humus nitrogen.....	3.6	3.4	4.9	5.7
Diamino nitrogen.....	26.2	32.8	30.0	30.0
Monamino nitrogen.....	64.8	60.3	60.2	60.3

In considering these figures it must be borne in mind that we are dealing with liver tissue from which not only the extractives, fats and lipoids have been removed, but also the greater part if not all the gelatigenous substances. Analysis of liver tissue by other observers in which these extractions have not been made are, it seems to me, of uncertain value, especially in pathological livers in which the great variation in fat as well as extractives and connective tissue can by themselves produce great alterations in the percentage figures, which are then incorrectly ascribed to the essential constituents of the liver cells themselves. Furthermore, it must be considered that the degree of regenerative proliferation and leucocytic invasion that is taking place in the liver will modify greatly the amount of purins and nucleoproteins. Taking these figures at their face value, however, they may be interpreted as meaning that the decrease in diamino nitrogen

which Wakeman<sup>1</sup> found to be so striking in the livers of dogs poisoned with phosphorus, and which was found to a less extent in my case of acute yellow atrophy, was not exhibited by the liver showing extensive necrosis from chloroform. More recently Wakeman<sup>2</sup> has analyzed a liver said to show acute yellow atrophy, in which there was found no decrease in the nitrogen of the bases. To draw any conclusions from these isolated observations, however, would not be warranted.

Determination of insoluble sulphur, phosphorus and iron in the extracted residues of these livers, diseased and normal, gave results that are difficult of interpretation. The following table gives the percentage amounts of these inorganic elements in the residues:

	Acute atrophy.	Normal (anemic).	Normal (congested).	Chloroform necrosis.
Sulphur.....	0.82	0.75	0.77	0.79
Phosphorus*.....	0.90	0.27	0.21	0.90
Iron.....	1.22	0.2	0.4	0.5

\* Average of four analyses of each specimen.

While the sulphur is practically constant in amount in all four specimens, in spite of the great structural changes in the two diseased livers, the insoluble phosphorus in each of the latter is increased to about four times the amount present in the normal livers. The increase in the phosphorus in the acute yellow atrophy may be readily explained as the result of the great proliferative activity exhibited by the cells of the stroma and bile ducts in areas where regeneration is taking place, which causes the presence of large numbers of new cells rich in nucleic acid. No such explanation is available for the increase of phosphorus in the chloroform necrosis liver, however, for in this specimen there is not only no proliferation, but also by far the majority of hepatic nuclei have disappeared, making it doubly hard to account for this decided increase of insoluble phosphorus. It is barely possible that some of the lecithin phosphorus has been so fixed

<sup>1</sup> *Journ. of Exper. Med.*, vii, p. 292, 1905.

<sup>2</sup> *This Journal*, iv, p. 119, 1908.

that it cannot be extracted from the cells, in view of the fact that there has been some loss of lecithin in both livers, but it is not probable that such a change could account for more than a small fraction of the increase in phosphorus that was observed. In any case these figures show that necrosis of the liver cells, with disappearance of the majority of stainable nuclei, is not necessarily associated with a decrease in the amount of insoluble phosphorus as would be expected.

In agreement with the histological picture, the strikingly large amount of gelatin obtained from the acute yellow atrophy liver as a result of the connective tissue proliferation present in this condition was not found in the liver of chloroform necrosis. While the acute yellow atrophy liver yielded 13.8 grams of gelatin or 10.1 per cent of the dry, fat-free tissue, a normal liver yielded but 3.2 per cent of the dry fat-free tissue as gelatin, and in the chloroform necrosis but 1.5 per cent was gelatin.

There was no such increase of the proportion of water in the liver as is found constantly in acute yellow atrophy, as shown in the following table:

	Water.	Fat.	Fat-free dried substances.
Normal liver (Quinke).....	76.1	3.0	20.9
Normal liver (Wells).....	77.6	5.0	17.4
Acute atrophy (Perls).....	81.6	8.7	9.7
"    "    (Perls).....	76.9	7.6	15.5
"    "    (v. Starek).....	80.5	4.2	15.5
"    "    (Taylor).....	85.8	2.0	12.2
"    "    (Wakeman*).....	79.3		
"    "    (Wells).....	83.8	2.5	13.7
"    "    (Voegtlin†).....	78.0	6.6	15.4
Phosphorus poisoning (v. Starek).....	60.0	29.8	10.0
Fatty degeneration (v. Starek).....	64.0	25.0	11.0
Chloroform necrosis (Wells).....	72.4	8.8	18.8

\* This *Journal*, iv, p. 119, 1908.

† *Johns Hopkins Hospital Bull.*, xix, p. 50, 1908.

The chloroform necrosis liver stands between the typical acute yellow atrophy liver and the ordinary fatty liver, according to its analytic figures as well as according to its histology. That

is, there has been some replacement of water by fats, but not so much replacement of protein by water as in acute atrophy. These figures emphasize the fact that in acute yellow atrophy there is no increase of fat in the liver, and that in chloroform necrosis the amount of fat is distinctly increased, although not so much is found chemically as might be expected from the microscopic findings.

The amount of lecithin and cholesterin in this liver is by no means so greatly altered from normal as was found to be the case in acute yellow atrophy, as shown by the following table:

	LECITHIN.				CHOLESTERIN.			
	Normal anemic.	Normal congested.	Chloroform necrosis.	Acute atrophy.	Normal anemic.	Normal congested.	Chloroform necrosis.	Acute atrophy.
Per cent								
of fresh weight . . . .	1.6	1.4	1.5	0.45	0.26	0.37	0.52	0.3
of total dry weight . .	6.3	6.25	6.2	2.9	1.0	1.7	1.9	1.8
of dry fat-free ma- terial . . . . .	7.7	8.0	8.1	3.2	1.25	2.1	2.9	2.1
of ether-soluble sub- stances . . . . .	35.3	28.0	17.3	17.6	5.7	7.4	5.9	11.1
entire liver . . . . .	23.7	22.4	16.0	4.4	3.8	5.95	5.4	3.38

While the total amount of lecithin has decreased, this is only in proportion to the decrease in the total size and weight of the liver; this proportional decrease has gone on in spite of a relative increase in the amount of simple fat, showing the same lack of correlation between the lecithin and the neutral fat which has been observed by others who have determined the lecithin content of organs showing fatty degeneration. Evidently, therefore, the increase in the fat content of the liver in chloroform necrosis is due entirely to simple fats. The cholesterin, on the other hand, has apparently remained in about the normal amount, and has not decreased with the lecithin and proteins; this is quite what might be expected from what we know of the tendency of cholesterin that is liberated by degenerating cells to remain at the place where it is formed.

The only other analysis of a liver showing chloroform necrosis that has been recorded in the literature is the one published by A. E. Taylor.<sup>1</sup> This case was very similar to the one described above, both clinically and anatomically. The liver weighed 1200 grams, was very soft, friable, and "putty-like," showing microscopically widespread degeneration of the liver cells. It contained over 200 grams of fat, and from the extracts were obtained 4 grams of leucin, 2.2 grams of tyrosin, and 2.3 grams of arginin nitrate. The other constituents of the liver were not determined.

These two analyses corroborate one another in showing the presence of free amino acids in amounts large enough for identification in the liver of chloroform necrosis. The amino acids are presumably derived from autolysis of the liver cells, although it is by no means certain that part of the free amino acids found in the liver may not have come from some other source.

#### SUMMARY.

In the necrosis of the liver which occasionally follows chloroform anesthesia there is a rapid autolysis of the liver cells, resulting in a loss of as much as one-third or more of the solids in three or four days, and indicated chemically by the presence of free amino-acids, purins, proteoses, peptones and polypeptids in the liver. Several of the amino-acids were present in quantities large enough to permit of their isolation and identification. Despite the loss of nearly all the nuclear structures of the liver the amount of insoluble phosphorus was found in the specimen examined to be increased, without alteration in the amount of insoluble sulphur. The distribution of the nitrogen as mono- and diamino acids in the insoluble coagulated liver proteins is not different from that of the proteins of the normal liver. There is a moderate degree of fatty metamorphosis, the microscopic and chemical findings corresponding in this respect; this increase in ether-extractive material being due to infiltration of simple fats, while there is a slight decrease in the lecithin and no alteration in the amount of cholesterin. There is less replacement of proteins by water and more fatty infiltration than in acute yellow atrophy.

<sup>1</sup> *Univ. of Calif. Publ. (Pathol.)*, i, 43, 1904.



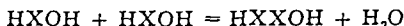
# ON THE INFLUENCE OF TEMPERATURE UPON THE SOLUBILITY OF CASEIN IN ALKALINE SOLUTIONS.

By T. BRAILSFORD ROBERTSON.

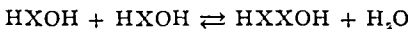
(From the Rudolph Spreckels Physiological Laboratory of the University of California.)

(Received for publication, June 10, 1908.)

In previous papers<sup>1</sup> I have suggested that a solution of a protein may be regarded as a system of polymeric modifications of the amphoteric electrolyte  $HXOH$ , the point of equilibrium being shifted by any variation in the conditions, such as the addition of acid, alkali, salts or the application of heat, which influences the concentrations of the protein ions; just as the simplest possible amphoteric electrolyte, namely, water, consists of a mixture of polymeric modifications of the molecule  $HOH$ , the point of equilibrium being shifted by alterations in temperature.<sup>2</sup> From this point of view the process of heat-coagulation would be regarded somewhat as follows; by repeated condensations of the type



larger and larger molecule-complexes are formed until the molecular aggregates assume the properties of matter in mass and the solution assumes the character of a suspension which is usually unstable, the protein particles being thrown out of solution in the form of coagula or flocculi. If this point of view be correct then it follows that one of the effects of applying heat to a protein solution must be the shifting of an equilibrium of the type:



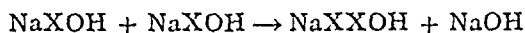
towards the right; were this so it would follow from van't Hoff's "principle of mobile equilibrium" that the hydrolysis of pro-

<sup>1</sup> T. Brailsford Robertson: *Journ. of Physical Chem.*, x, p. 524, 1906; xi, p. 453, 1907; *This Journal*, iv, p. 23, 1908.

<sup>2</sup> Svante Arrhenius: *Text-book of Electro-chemistry*, Trans. by John McCrae, p. 116, 1902; Wm. Sutherland: *Phil. Mag.*, 1, p. 460, 1900.

teins is accompanied by the evolution of heat, which conclusion is in complete accord with experimental observation in so far as positive results have been obtained.<sup>1</sup> The hypothesis is furthermore in accord with the view that heat-coagulation is accompanied by the withdrawal of water from the protein.<sup>2</sup> Views in many respects similar to these have been expressed by a number of authors. Mann, indeed, states that in his opinion heat-coagulation is "brought about by one portion of the albumin molecule precipitating the remainder," a view which is essentially similar to that expressed above.<sup>3</sup> In this connection it is also of interest to note that many authors have considered that the initial stages of protein hydrolysis consist in the "depolymerization" of the protein molecule.<sup>4</sup> Sutherland has also pointed out that the weight of different proteins which combines with a gram equivalent of a heavy metal is a simple multiple of the lowest observed weight and he deduces therefrom that there is a large amount of "internal salt" formation in proteins; he has also expressed the view, practically identical with that put forward above, that coagulation of a protein is the result of polymerization through the neutralization of "valencies which are usually latent."<sup>5</sup>

If the heat-coagulation of a protein consists in the polymerization of the amphoteric protein molecule with the elimination of water, according to the equation given above, then the influence of heat upon a compound of a protein with a base should be of the following type:




---

<sup>1</sup> The heat of reaction of protein hydrolysis is extremely small, observers have either failed to detect any change in the heat-content of the system or else have observed a very slight *disengagement* of heat. Tangl: *Arch. f. d. ges. Physiol.*, cxv, p. 1, 1906; v. Lengyel: *Ibid.*, p. 7; Hari: *Ibid.*, p. 11, *Ibid.*, cxxi, p. 459, 1908.

<sup>2</sup> Michailow: *Chem. Centralb.*, p. 1088, 1887; Starke: *Zeitschr. f. Biol.*, Jubelband z. Ehren v. C. Voit, p. 206, 1901.

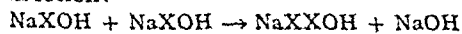
<sup>3</sup> Gustav Mann: *Chemistry of the Proteids*, London, p. 318, 1906.

<sup>4</sup> Maly: *Arch. f. d. ges. Physiol.*, ix, p. 585, 1874; xx, p. 315, 1879; Herth: *Zeitschr. f. physiol. Chem.*, i, p. 277, 1878; Poehl: *Ber. d. deutsch. chem. Gesellsch.*, p. 1355, 1881; p. 1152, 1883; Loew: *Arch. f. d. ges. Physiol.*, xxxi, p. 393, 1883.

<sup>5</sup> Wm. Sutherland: *Proc. Roy. Soc.*, London, p. 130, 1906.



and the solution of the compound should become more alkaline on heating. The marked increase in the alkalinity of solutions of the caseinates of bases, which occurs on heating, has been observed by Osborne,<sup>1</sup> but his interpretation of the phenomenon is quite different to that which is suggested above. He considers that the action of heat consists in increasing the hydrolytic dissociation of the caseinate. Since the free casein is very insoluble and, presumably, very slightly dissociated, an increase in the degree of hydrolytic dissociation of the salt would lead to an increase in the alkalinity of the solution, and furthermore, might be reasonably expected to lead to a marked opalescence of the solution or even to the precipitation of casein, since the free casein is insoluble in water. A marked increase in opalescence, on heating to 35° to 45° C. was observed by Osborne in solutions of calcium, barium, magnesium and lithium caseinates but it was not observed in solutions of sodium, potassium or ammonium caseinates; if the opalescence were due to undissociated casein being set free by hydrolytic dissociation it is difficult to see why it does not occur in solutions of sodium and potassium caseinate and especially in solutions of ammonium caseinate in which, as Osborne himself points out, since the ammonium hydroxide is a very weak base, hydrolysis might be expected to be especially intense. It is, however, possible to decide between the two hypotheses in a very simple way. I have shown in a previous paper<sup>2</sup> that a given amount of alkali dissolves just sufficient casein to form the "neutral caseinate" of the base (such that 8 cc. of  $\frac{N}{10}$  alkali = 1 gram of casein) and that the resulting solution is neutral to litmus. If the influence of the application of heat upon this solution consisted in increasing the hydrolytic dissociation of the caseinate it would follow that the power of the given amount of alkali to bind casein is diminished by heat and therefore the solubility of casein in a given concentration of alkali would be diminished by an increase in temperature; on the contrary if the influence of the application of heat upon a solution of a neutral caseinate consists in the shifting of the equilibrium of the system in the direction:



<sup>1</sup> W. A. Osborne: *Journ. of Physiol.*, xxvii, p. 398, 1901.

<sup>2</sup> T. Brailsford Robertson: *This Journal*, ii, p. 317, 1907.

then the alkali set free should be capable of dissolving more casein, or in other words, the solubility of casein in a given concentration of alkali would be *increased* by an increase in temperature. From either hypothesis the increase in the electrical conductivity of the solution upon heating, which was observed by Osborne, would necessarily follow. The following experiments were undertaken with a view to ascertaining which of the alternative hypotheses represents the facts more accurately.

#### EXPERIMENTAL.

It has been pointed out by Osborne, in the paper referred to above, that the influence of temperature upon solutions of caseinates is reversible, i. e., that the opalescence and increase in alkalinity which appear on heating disappear on cooling and reappear on heating again. This is probably true for all heat coagulations but where the protein is thrown down in coagula the hysteresis of the system (owing to the excessive internal molecular friction of large aggregates) prevents its reattaining equilibrium with a measurable velocity on cooling. That the equilibrium characteristic of low temperatures is rapidly regained if heat-coagulation is not pushed too far, so that the molecular aggregates which are formed are not too large, has been shown by Corin and Ansiaux<sup>1</sup> who find that the first traces of coagulation disappear on quickly cooling and shaking the solution. We may, therefore, assume that a solution of a caseinate which has been heated regains, on cooling, its original condition and power of neutralizing bases. The method of procedure was as follows. Five, ten etc., cc. of  $\frac{N}{10}$  potassium or lithium hydrate or of a saturated solution of calcium hydrate (approximately  $\frac{N}{20}$ ), were diluted to 100 cc. with distilled water, placed in tightly stoppered Erlenmeyer flasks and warmed in a thermostat to the desired temperature. Three times the amount of casein which would be dissolved by the given amount of alkali at room temperature (i. e., 3 grams to every 5 cc. of  $\frac{N}{10}$  alkali) was then introduced and the mixture left in the thermostat for from thirty to forty minutes, being vigorously shaken at frequent intervals. The resulting solution was then filtered in the thermostat (the filter and the receiving vessel having been previously warmed to the desired

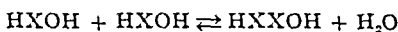
<sup>1</sup> Corin and Ansiaux: *Bull. de l'acad. roy. de Belg.*, No. 21.

temperature) and the filtrate was allowed to cool or was cooled by immersing the containing vessel in tap-water. The temperatures were defined to within  $\frac{1}{2}^{\circ}$ . An aliquot part (25 cc.) of the solution was then titrated against the alkali which had been used to dissolve the casein, phenolphthalein being used as indicator. Since 8 cc. of  $\frac{N}{10}$  alkali neutralize one gram of casein to phenolphthalein<sup>1</sup> if the amount of alkali originally contained in the volume of solution titrated is known and the amount which it is necessary to add in order to secure neutrality to phenolphthalein is ascertained the amount of casein contained in the solution can immediately be deduced from the relation 1 cc.  $\frac{N}{10}$  alkali = .125 gram of casein. In a previous paper I have shown that this method of determination yields reliable results.<sup>2</sup>

The following were the results obtained:

Concentration of the alkaline solution.	Grams of casein dissolved per 100 cc. solution at—							
	21°	36°	46°	54°	60°	66°	81°	88°
$23 \times 10^{-4}$ N KOH	0.46					0.52		
$46 \times 10^{-4}$ "	0.92	0.92	1.04		1.34	1.27	1.34	1.13
$69 \times 10^{-4}$ "	1.38					2.11		
$92 \times 10^{-4}$ "	1.85			2.77		3.05		
$44 \times 10^{-4}$ LiOH	0.89	0.86			1.28	1.14	1.60	1.20
$88 \times 10^{-4}$ "	1.77			2.62		2.95		
$45 \times 10^{-4}$ Ca(OH) <sub>2</sub>	0.90	0.72			0.65	0.63	0.64	0.63
$90 \times 10^{-4}$ "	1.80			1.35				

It is evident that the power of the bases, potassium hydroxide and lithium hydroxide, to dissolve casein is greatly increased by increasing temperature. In all cases, the solutions, at the temperatures indicated, were acid to phenolphthalein and approximately neutral to litmus so that the effect of heating a solution of a caseinate cannot be to increase its hydrolytic dissociation. The facts are much more readily explained on the supposition that the effect of temperature consists in shifting the equilibrium:



<sup>1</sup> Van Slyke and Hart: *Amer. Chem. Journ.*, xxxiii, p. 461, 1905; T. Brailsford Robertson: *This Journal*, ii, p. 317, 1907.

<sup>2</sup> T. Brailsford Robertson: *This Journal*, ii, p. 317, 1907.

## 152 Influence of Temperature upon Solubility of Casein

towards the right so that a given amount of alkali, since it is associated with a molecule of nearly double the weight, neutralizes nearly twice as much casein at  $66^{\circ}$  as it does at room temperature ( $21^{\circ}$ ). The marked *diminution* in the solubility of casein in calcium hydrate solutions which occurs on raising the temperature can be explained by supposing that the salt  $\text{Ca}(\text{XXOH})_2$  is insoluble while the salt  $\text{Ca}(\text{XOH})_2$  is soluble, and this explains also why Osborne obtained an increase in opalescence upon heating solutions of calcium, magnesium and barium caseinates but not upon heating solutions of potassium, sodium and ammonium caseinates. Since calcium hydrate solutions do not fail, at any of the temperatures investigated, to dissolve *some* casein it is evident that the conversion of the casein molecule  $\text{HXOH}$  into double, triple or higher polymers cannot be complete but that an equilibrium between the two forms exists at every temperature. Since calcium hydrate solutions of different concentrations dissolve different amounts of casein at the same temperature (as can be deduced by interpolation from the above table) the amount which is dissolved by any given concentration of calcium hydrate cannot represent, merely, saturation of the solution with the salt  $\text{Ca}(\text{XXOH})_2$  or  $\text{Ca}(\text{XXXOH})_2$ , but must represent also the solution of the calcium salt of unpolymerized molecules in equilibrium with a saturated solution of the calcium salt of the polymerized molecules. It appears probable in the light of these results, that the view which I have previously expressed is the correct one, namely, that the influence of heat upon proteins consists, among other effects, in shifting the equilibrium among the polymeric modifications of the amphoteric protein molecule in the direction of higher complexes.

An alternative hypothesis, which would cover the above facts, is that casein acts as a dibasic acid and that at room temperatures salts of the type  $\text{Na}_2\text{X}(\text{OH})_2$  are formed while at higher temperatures acid salts of the type  $\text{NaHX}(\text{OH})_2$  are formed. The fact that solutions of both the neutral and "basic" caseinates obey Ostwald's dilution-law for a salt of a monobasic acid, however, excludes this possibility.<sup>1</sup> A possible source of error in the above experi-

<sup>1</sup> T. Brailsford Robertson: *Journ. of Physical Chem.*, xi, p. 542, 1907; *Ibid.* (shortly to appear).

mental determinations may be mentioned. Solutions of the neutral caseinates undergo fairly rapid auto-hydrolysis, about one-third being hydrolyzed in twelve hours at 37°.¹ This effect would of course be negligible in the short period during which the solutions are being prepared, but at higher temperatures the velocity of hydrolysis would probably be increased and this might conceivably vitiate the accuracy of the titrations. Special determinations made with a view to estimating the magnitude of the error thus introduced showed that it was nearly inappreciable. Thus 100 cc. of  $46 \times 10^{-4} N$  KOH at 88° dissolves 1.13 gram of casein, the solution having been allowed to stand in the thermostat for one-half hour. After *three* hours in the thermostat the titration indicated that 1.25 gram had been dissolved. The error at 88° in half an hour would therefore be, in this solution, about .04 gram and at lower temperatures and in more dilute solutions it must, of course, be considerably less. It may here be noted that in none of the solutions in which more casein was dissolved at higher temperatures than would be dissolved at room temperature was any appreciable tendency towards precipitation of casein on cooling observed, although in many cases there was a marked increase in the opalescence of the solution. This is, however, not surprising since an appreciable amount of acid may be added to a solution of alkali "saturated" with casein at room-temperature before precipitation occurs. Such solutions are possibly to be regarded as being "super-saturated" with casein and in a condition of unstable equilibrium.

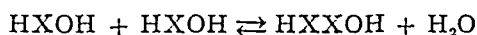
#### CONCLUSIONS.

- (1) The solubility of the casein in alkaline solutions is considerably augmented by carrying out the process of solution at temperatures above 40° C.
- (2) It is pointed out that this fact is not in harmony with the view that a rise in temperature increases the degree of hydrolytic dissociation of solutions of the caseinates.
- (3) In explanation of this fact and of the increase in alkalinity and electrical conductivity of caseinate solutions upon heating,

¹ T. Brailsford Robertson: This *Journal*, ii, p. 317, 1907.

## 154 Influence of Temperature upon Solubility of Casein

which were observed by Osborne, it is suggested that the influence of heat upon proteins consists, among other effects, in shifting equilibria of the type:



in the direction of higher complexes, and that heat-coagulation is a result of repeated condensations of this type.

(4) The solubility of casein in solutions of various concentrations of potassium hydroxide, lithium hydroxide and calcium hydroxide at various temperatures has been determined.

## NOTE ON THE APPLICABILITY OF THE LAWS OF AMPHOTERIC ELECTROLYTES TO SERUM GLOBULIN.

By T. BRAILSFORD ROBERTSON.

(From the Rudolph Spreckels Physiological Laboratory of the University of California.)

(Received for publication, June 3, 1908.)

In a recent number of this *Journal*, H. Lundén<sup>1</sup> has raised a number of objections to statements and computations made by me in my paper on the dissociation of serum globulin at varying hydrogen ion concentrations.<sup>2</sup> While in dealing with his objections, I should have preferred to bring forward additional experimental matter, yet as pressure of other work forbids this just at present and as Dr. Lundén's statements are, in many cases, very misleading, I have thought it advisable not to delay publication of the following short review of some of the more important points raised by him. The first of my statements to which Lundén takes exception is that to the effect that the method which has frequently been used to determine the dissociation-constants of amphoteric electrolytes, namely, by estimating the hydrolysis of a salt and proceeding to calculate the dissociation-constant in the usual manner for non-amphoteric electrolytes "can only give even approximately accurate values for the larger function if it be sufficiently large compared with the other, otherwise the dissociation-constants obtained in this way are subject to considerable error," and he proceeds to demonstrate that the method can be used even for determining the smaller constant without appreciable error. That an error is introduced by this procedure, however, he admits, and he proceeds to estimate the magnitude of the error under varying conditions. The computations thus tabulated by Dr. Lundén are of considerable interest and value and I am gratified that my imperfectly accurate statement should have elicited them. It is

<sup>1</sup> H. Lundén: This *Journal*, iv, p. 267, 1908.

<sup>2</sup> T. Brailsford Robertson: *Journal of Physical Chem.*, xi, p. 437, 1907.

a question, however, to what extent my objection to the method has been invalidated by Dr. Lundén's computations in so far as the particular problem under consideration is concerned, namely, the equilibrium of proteins in acid or alkaline solution. Lundén estimates that if the dissociation-constant which is being determined is less than  $10^{-9}$  while the other dissociation-constant is less than  $10^{-5}$  and the dilution is less than 100 then the correction which must be applied to the constant as determined by the usual method is less than 1 per cent. As he points out, however, at dilutions greater than this or if the dissociation-constant which is not being determined is *greater* than  $10^{-5}$  then the correction may assume considerable importance and he illustrates this fact by referring to the case of aspartic acid. Now equilibria in protein systems are, of necessity, usually investigated at high dilutions and consequently the correction may, in these cases, be expected to be of appreciable magnitude. Moreover, Dr. Lundén's statement (p. 280 of his paper referred to above) that cases in which one of the constants is greater than  $10^{-5}$  are not likely to occur among the proteins betrays a lack of familiarity with these bodies. Thus casein is practically insoluble in water<sup>1</sup> yet a suspension of casein in water will displace carbonic acid from carbonates.<sup>2</sup> Since the molecular concentration of dissolved casein, under these conditions, must be exceedingly minute casein must be a considerably stronger acid than carbonic acid. Serum globulin, judging by its general behavior, cannot be far inferior in strength to casein while some of the mucins and nucleic acids are not improbably stronger.

In my paper upon serum globulin the various symbols employed have the following significance:

The protein molecule ( $= \text{HXOH}$ ) is in equilibrium with the acid  $\text{HCl}$ , in the solution we have the following ions and molecules in the concentrations mentioned below:

$\text{H}^+$	$\text{OH}^-$	$\text{XOH}^-$	$\text{HX}^+$	$\text{HXOH}$	$\text{XX}$	$\text{Cl}^-$	$\text{HXCi}$
<i>a</i>	<i>b</i>	<i>c</i>	<i>d</i>	<i>e</i>	<i>f</i>	<i>r</i>	$\mu$

<sup>1</sup> T. Brailsford Robertson: *This Journal*, ii, p. 317, 1907; van Slyke and van Slyke: *Amer. Chem. Journ.*, xxxviii, p. 383, 1907; *this Journal*, iv, p. 259, 1908.

<sup>2</sup> W. A. Osborne: *Journ. of Physiol.*, xxvii, p. 398, 1901.



The ionic velocities of the  $H^+$  and  $Cl^-$  ions in cm-sec. are written, respectively,  $U$  and  $V$  while that of the protein ions (assumed to be equal for the positive and negative ions) is written  $v$ . The total amount of hydrochloric acid present, combined and uncombined, is written  $a_1$  while the quantity  $a_1 - a$  which is the amount of acid neutralized (determined by the gas-chain) is written  $m$ . The difference between the measured conductivity of the solution and the calculated conductivity of the uncombined HCl is written  $\lambda$  while the constant quantity

$$\frac{V - v}{V + v} \frac{K_w k_a}{k_b}$$

is written  $H$ .

On p. 281 of the paper referred to Lundén states that "from the equations [16], [13] and [10] in the paper of Robertson it follows that:

$$\left\{ \begin{array}{l} a_1 - a = m = \frac{\lambda}{V + v} - \frac{\frac{2v}{V^2 - v^2} H \lambda}{a^2 - H} = d - c \\ \mu = m - d + c^1 \\ \text{Hence:} \\ \mu = 0 \\ \text{and} \\ \gamma = a + d - c = a + a_1 - a = a_1 \end{array} \right.$$

Robertson consequently makes the supposition that the salt is completely ionized ( $\mu = 0$ ) and he also makes the supposition that the concentration of the  $Cl^-$  ion is equal to the total concentration of Cl ( $\gamma = a_1$ ). These are the same suppositions as I have made in the former part of this paper."

Dr. Lundén is evidently endeavoring to convey the impression that I have overlooked or omitted to mention certain assumptions hidden in my equations. Were Lundén's quotation accurate this would certainly be the case but it is not and although criticism is welcome I must beg leave to protest against misquotation.

<sup>1</sup> In Lundén's paper this equation is erroneously written  $\mu = m - d - c$ .

The statements actually made in my paper (p. 440) are as follows:

$$m = \frac{\lambda}{V + v} - \frac{\frac{2v}{V^2 - v^2} H \lambda}{a^2 - H} + \frac{J a^2 \lambda \left( a + \frac{\lambda}{V + v} - \frac{\frac{2v}{V^2 - v^2} H \lambda}{a^2 - H} \right)}{a^2 - H}$$

In *practice* it is found that the term:

$$\frac{J a^2 \lambda \left( a + \frac{\lambda}{V + v} - \frac{\frac{2v}{V^2 - v^2} H \lambda}{a^2 - H} \right)}{a^2 - H}$$

is negligible, when the ampholyte is chiefly acid, except at comparatively high concentrations of the acid HA (HCl), so that for low concentrations of the acid HA (HCl) we have:

$$m = \frac{\lambda}{V + v} - \frac{\frac{2v}{V^2 - v^2} H \lambda}{a^2 - H} ,,$$

This latter equation being the one quoted by Lundén. It will be seen that the term which *experiment* showed could be rejected is equal to  $\mu$  (by substituting from equations [10] and [11] in my paper) but at no step in my analysis was the *supposition* made that it was equal to zero: on the contrary, for the values of the constants given in my paper it was an experimentally ascertained fact that the value of  $\mu$  was very small and it is Lundén who makes the *supposition* that it is negligible and not I. In fact in Table III of my paper I evaluate  $\mu$  for the various dilutions employed; this would hardly have been a consistent procedure on my part I had previously *assumed* that  $\mu$  was zero.

In what follows I cannot subscribe to Dr. Lundén's logic. Adopting the value for  $v$  which I found to fit the equations evaluated above and assuming that it is correct he proceeds to demonstrate, by its help, that these equations do not apply to the system under consideration; later on he uses my constant  $\frac{K_w k_a}{k_b}$  for the same purpose; but if, as Dr. Lundén supposes,

the above equations are not applicable to this system then the constants deduced by employing them certainly cannot be accurate and Dr. Lundén is not justified in assuming that they are correct in order to demonstrate that the equations are erroneous. It is not necessary, however, to impugn Dr. Lundén's logic in order to account for certain of the contradictions which he has discovered between my experimental data and his equations. If Dr. Lundén will peruse the ninth line of print on p. 446 of my paper he will discover that in applying equation [21] of his paper he has equated quantities determined at 25° to quantities determined at 18°; this being the case it is not improbable that Dr. Lundén's statement that "the values of  $d$  and  $c$  calculated from equations [21] and [23] are all absurd" will meet with general acceptance. As a matter of fact Lundén's equation [21] cannot, with accuracy, be directly applied to these observations because although we know with considerable accuracy the temperature-coefficient of the conductivity of solutions of hydrochloric acid we do not, with such accuracy, know the temperature-coefficient of the ionic velocity of  $\text{Cl}^-$  nor the manner in which this depends upon the concentration of the solution. In the equations which I employed, on the contrary, since the only temperature-coefficient which had to be introduced was that for hydrochloric acid of concentration =  $a$  the introduction of the correction for temperature involved no appreciable inaccuracy.

Dr. Lundén further endeavors, using the value given in my paper for the constant  $\frac{K_w k_a}{k_b}$  to apply the following equations to the system:

$$\frac{d}{c} = \frac{a^2 k_b}{K_w k_a}$$

$$d - c = a^2 - a$$

and obtains values for  $d$  and  $c$  which, although closely commensurate with those given by me in Table VI of my paper are nevertheless negative for the lower concentrations of  $\text{H}^+$  ions; from this fact, which owing to the method of calculation adopted by me, had escaped my notice, and from the fact that his equations [21] and [23] do not yield values commensurate with those

obtained from these equations Dr. Lundén concludes that "the formulæ used by Robertson do not represent the chemical equilibria in globulin solutions." Two alternative possibilities, however, which do not appear to have suggested themselves to Dr.

Lundén are that my value for the constant  $\frac{K_w k_a}{k_b}$  is too large or that the observation in question is inaccurate. A simple criterion enables us to decide which of these two alternatives is the correct one. From equation [13] of my paper it follows that in all cases  $\lambda$  must be greater than  $m(V + v)$  and since  $V + v$  cannot be less than  $V$  it follows that  $\lambda$  must always be greater than  $mV$ ; otherwise the conductivity of the solution would be less than the conductivity due to the  $H^+$  and  $Cl^-$  ions present in the solution, which is impossible. Now  $V$  at  $18^\circ$  is  $65.8 \times 10^{-5}$  cm-sec. and at  $25^\circ$  it cannot be less than this, hence in all cases  $\lambda$  must be greater than  $65.8 \times 10^{-5} m$ , that is, for the observation  $m = 14.9 \times 10^{-4}$  (for which Lundén finds negative values of  $d$  and  $c$ ) cannot be less than  $9.8 \times 10^{-7}$ , whereas the observation actually recorded is  $\lambda = 7.8 \times 10^{-7}$ ; hence this observation must be rejected; the remaining observations do not violate this criterion. Since the observation immediately following that rejected above is also untrustworthy (cf. p. 448 of my paper) there remain four observations which, as Lundén's and my computations show, obey the equations evaluated in my paper fairly accurately and lead to consistent and intelligible values of  $c$  and  $d$ . I do not consider, however, that four observations are sufficient to enable us to state that serum globulin obeys the laws for dissociation of an amphoteric electrolyte in other than a qualitative manner; still less do I consider them adequate to sustain the far-reaching conclusion of Dr. Lundén that the equations evaluated in my paper do *not* represent the chemical equilibria in globulin solutions.

Dr. Lundén objects to the endeavor to apply the laws for the dissociation of a monovalent amphoteric electrolyte to proteins because they contain more than one  $COOH$  or  $NH_2$  group. While this fact is undeniable it nevertheless does not follow that the laws which apply to the dissociation of a monovalent electrolyte cannot apply to the dissociation of these bodies. It is well known that many di- or tri-valent acids and bases behave,

in solution, essentially as if they were monovalent, the reason being that the second and third hydrogen (or hydroxyl) ions are split off with increasing difficulty and in indefinitely small quantities.<sup>1</sup> Thus succinic acid obeys Ostwald's dilution-law for a monovalent acid and in titrating against bases with methyl-orange indicator can also be regarded as monovalent. As a matter of fact I have shown that solutions of the neutral caseinates of ammonium and sodium obey Ostwald's dilution-law for a salt of a monovalent acid;<sup>2</sup> since, therefore, it has been definitely shown in the case of one protein that only one COOH group is appreciably concerned in determining the equilibrium of the system it is not illogical to assume, until experiment proves otherwise, that similar conditions prevail in the equilibria of other proteins.

In the latter part of his paper Lundén devotes some space to demonstrating that my estimates of the molecular weight of globulin from conductivity and gas-chain data can only be approximate in character. I think a perusal of my paper, referred to above, will make it clear that I was sufficiently aware of this fact and that the essentials of Dr. Lundén's criticism of these computations were adequately dealt with therein. Since unionized and uncombined serum globulin is only inappreciably soluble<sup>3</sup> the "internal salt" and the unhydrated form of the serum globulin could only be present in inappreciable quantities and Lundén's contention that their presence invalidates the determination of the molecular weight by these methods fails to apply to the case under consideration while it again reveals the author's lack of familiarity with the proteins. The error due to the "association" which the protein undergoes in solution is fully discussed in my paper.<sup>4</sup>

<sup>1</sup> Cf. E. E. Chandler: *Journ. of the Amer. Chem. Soc.*, xxx, p. 694, 1908, in which the literature is also given.

<sup>2</sup> T. Brailsford Robertson: *Journ. of Physical Chem.*, xi, p. 542, 1907.

<sup>3</sup> Clarence Quinan: *Univ. of Calif. Publ. Pathol.*, i, p. 1, 1903; Cf. also Gustav Mann: *Chemistry of the Proteids*, London, pp. 361, etc., 1906.

<sup>4</sup> It may here be noted that the potential difference between a hydrogen electrode in  $\frac{1}{10}$  N hydrogen ion solution and the calomel electrode (with  $\frac{1}{10}$  KCl) is, by a clerical error, given in my previous paper as .326 volt. It should of course, be .336 volt.



# X. RESEARCHES ON PYRIMIDINS: THE ACTION OF DIAZOBENZENE SULFONIC ACID ON THYMIN, URACIL AND CYTOSIN.

(Thirty-fourth Paper.)

By TREAT B. JOHNSON AND SAMUEL H. CLAPP.

(From the Sheffield Laboratory of Yale University.)

(Received for publication, June 9, 1908.)

We have, at the present time, practically no knowledge of the way in which the pyrimidins—thymin, uracil and cytosin—are linked in the nucleic acid molecule. The question whether they are actually as such contained in the nucleic acids, from which they are obtained, has not been settled. The recent work of Osborne and Heyl<sup>1</sup> on triticonucleic acid, and of Levene and Mandel<sup>2</sup> would seem to indicate that they do not result from the purin bases,<sup>3</sup> but that the pyrimidin nucleus is present in nucleic acids in the simple form.

In order to obtain new data, which might prove of service in settling the question of the nature of the linking of pyrimidins in nucleic acids, we undertook this investigation. We shall describe the behavior of diazobenzene sulfonic acid on thymin, uracil, cytosin and some of their alkyl derivatives.<sup>4</sup> A summary of the results of our experiments, and their significance, is given at the end of this paper.

Burian<sup>5</sup> has investigated the action of diazobenzene sulfonic acid on several nucleic acids and purins. He examined the nucleic acids from sperma, thymus, yeast and spermatozoa of the

<sup>1</sup> *Amer. Journ. of Physiol.*, xxi, p. 157.

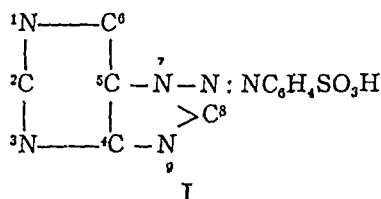
<sup>2</sup> *Biochem. Zeitschr.*, ix, p. 233.

<sup>3</sup> Asher-Spiro: *Ergeb. d. Physiol.*, v, p. 795, 1905; Burian: *Zeitschr. j. physiol. Chem.*, li, p. 438, 1907.

<sup>4</sup> Johnson and Clapp: *This Journal*, v, p. 49.

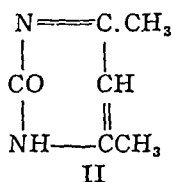
<sup>5</sup> *Ber. d. deutsch. chem. Gesellsch.*, xxxvii, p. 708; *Zeitschr. j. physiol. Chem.*, li, p. 435.

herring; and states that they do not react with this reagent. He showed, on the other hand, that the purins—xanthin, hypoxanthin, guanin, adenin and theophyllin—in which the hydrogen in position 7 is unsubstituted, react with diazobenzene sulfonic acid, in presence of alkali, giving intensely colored compounds. He regards the compounds formed as diazoamino derivatives of the general formula I. Substitution in the pyrimidin (alloxan)



ring apparently had no influence on the reaction. On the other hand, he observed that purins substituted in position 7—theobromin, caffein—and also uric acid, do not react with the diazo acid. Burian concludes from these results that the xanthin bases are linked in the nucleic acids at the nitrogen atom in position 7.

So far as the writers are aware Evans<sup>1</sup> was the first investigator to observe that a diazobenzene derivative reacts with a pyrimidin giving a colored compound. He found, for example, that 2-oxy-4,6-dimethylpyrimidin, II,



combines with diazobenzene chloride, in presence of alkali, giving a red compound. He says: "Es ist dies ein sehr kräftiger Farbstoff." The compound was unstable and no formula was assigned it.

Steudel<sup>2</sup> afterwards observed that natural thymin reacts with diazobenzene sulfonic acid, in alkaline solutions, giving an intense

<sup>1</sup> *Journ. f. prakt. Chem.*, xlviii, p. 489.

<sup>2</sup> *Zeitschr. f. physiol. Chem.*, xlii, p. 170.



red color. Pauly also mentions this diazo reaction in a later publication<sup>1</sup> and states that it cannot be used to distinguish between the pyrimidin and imidazol rings. He observed, for example, that 4-methyluracil gives as intense a color with diazobenzene sulfonic acid as histidin.

We now find that not only thymin and 4-methyluracil, but also uracil, 4,5-dimethyluracil, cytosin, 5-methylcytosin and 5-bromuracil react under proper conditions with diazobenzene sulfonic acid giving red colored solutions. The colors obtained with thymin, 4-methyluracil, 4,5-dimethyluracil and 5-methylcytosin are much more intense than those obtained with uracil, cytosin and 5-bromuracil. Apparently, the character of the groups occupying the 4 and 5 positions has a decided influence on the intensity of the color.

We have also made the interesting observation that the formation of red colors is entirely inhibited by substitution in the 3 positions of the uracil, cytosin and thymin molecules. The assumption that the diazo compound might react at the 4 position of the pyrimidin ring<sup>2</sup> giving azo derivatives is excluded by Pauly's observation<sup>3</sup> and also by the fact that 4, 5-dimethyluracil reacts with the diazo acid giving a red color. Substitution in position 1 of thymin does not prevent the formation of a red color. 1-Methylthymin,<sup>4</sup> for example, gave as intense a color as thymin itself. On the other hand a red color was not obtained with the diazo acid when the hydrogens in positions 1 and 3 or 3 alone of thymin were substituted by methyl groups.

Similar observations were also made in the cases of uracil and 5-bromuracil. While these pyrimidins gave red colors with the diazo acid, no colors were obtained when 1,3-dimethyluracil<sup>5</sup> and 3-methyl-5-bromuracil were tested with the reagent. The corresponding 3-methyluracil is unknown. The formation of a color in the cases of cytosin, III, and 5-methylcytosin, VI, is not dependent upon the presence of the free amino radical. 2-Oxy-

<sup>1</sup> *Zeitschr. f. physiol. Chem.*, xlii, p. 512.

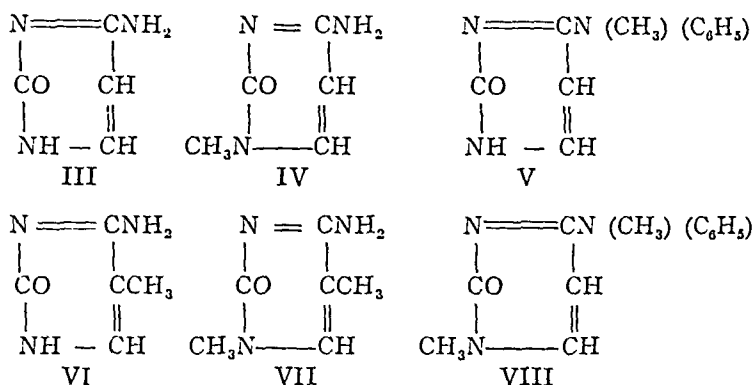
<sup>2</sup> Burian: *Zeitschr. f. physiol. Chem.*, xlii, p. 297; Mann's *Chemistry of the Proteids*, p. 431, 1906.

<sup>3</sup> *Loc. cit.*

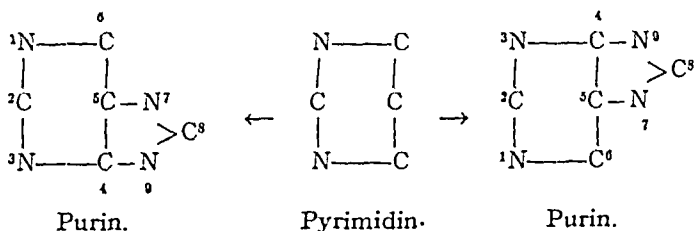
<sup>4</sup> Johnson and Clapp: *Loc. cit.*

<sup>5</sup> *Ibid.*

6-methylphenylaminopyrimidin V,<sup>1</sup> gave as intense a color as cytosin, III, while, on the other hand, no color was obtained with 3-methylcytosin, IV, 3,5-dimethylcytosin, VII, and 2-oxy-3-methyl-6-methylphenylamino-pyrimidin, VIII.



The remarkable tendency of certain pyrimidins to react with diazobenzene sulfonic acid, and the apparent inertness of the same ring in purins are of especial interest. According to Burian<sup>2</sup> substitution in the pyrimidin nucleus of purins did not influence the reaction with the diazo acid. While purins are closely related in structure to pyrimidins it is important to point out in this connection that positions 1 and 3 of the pyrimidin ring do not necessarily correspond to positions 1 and 3 in purins. While the pyrimidin nucleus is symmetrical, the purin, on the other hand, is unsymmetrical with respect to the 2 position. Two purins can theoretically be formed from a pyrimidin ring according as the glyoxalin ring is joined at the 4, 5 or 5, 6 positions. An inspection of the formulas below will show that this involves a change in the numbering of the atoms in the pyrimidin nucleus.



<sup>1</sup> Johnson and Clapp: *Loc. cit.*

<sup>2</sup> *Loc. cit.*

It is also of interest to note here that 6-oxypyrimidin<sup>1</sup> and 6-aminopyrimidin<sup>2</sup> do not give a red color with diazobenzene sulfonic acid. Whether this reagent reacts to form colored compounds only with pyrimidins having a —CO-NH— grouping in the 1, 2 or 2, 3 positions must be decided by further experiments.

The compounds formed by the action of diazobenzene sulfonic acid or pyrimidins appear to be more unstable than those formed in the case of purins. Two attempts to isolate the reaction-product in the case of thymine were unsuccessful.

The diazo acid used in our work was prepared according to the directions of Pauly.<sup>3</sup> That the test shall not be found to be capricious it is absolutely necessary that the acid be pure and freshly prepared.

#### METHODS OF APPLYING THE TEST.

##### 1. *With 10 per cent sodium hydroxide solution.*

Five to ten milligrams of the pyrimidine are dissolved in 0.5 cc. of 10 per cent sodium hydroxide solution and about 5.0 milligrams of diazobenzene sulfonic acid then added to the solution. If no reaction takes place the solution usually assumes a yellow or orange color. When a red color is formed it usually develops quite rapidly. The red color is usually quite permanent, lasting in some cases (thymine) for several hours.

##### 2. *With $\frac{N}{10}$ sodium hydroxide solution.*

Dissolve 5.0 to 10.0 milligrams of the pyrimidine in 2 cc. of  $\frac{N}{10}$  sodium hydroxide solution and add 5.0 to 10.0 milligrams of the sulfonic acid. This method of testing is not as reliable as Method 1.

##### 3. *Testing on a watch glass.*

Five to ten milligrams of the pyrimidine and an equal weight of the sulfonic acid are mixed together, with a glass rod, on a dry

<sup>1</sup> Wheeler: *This Journal*, iii, p. 285, 1907.

<sup>2</sup> Büttner: *Ber. d. deutsch. chem. Gesellsch.*, xxxvi, p. 2232, 1903; Wheeler: *Loc. cit.*

<sup>3</sup> *Zeitschr. f. physiol. Chem.*, xlii, p. 516.

watch glass. A drop of 10 per cent sodium hydroxide solution is then allowed to flow into the mixture. The red color develops immediately under these conditions. This method of applying the diazo test is recommended on account of its delicacy and reliability.

The results of our experiments are given, for comparison, in the following tables:

TABLE I.

Pyrimidins.	0.5 cc. 10 per cent NaOH solution.	2.0 cc. $\frac{N}{10}$ NaOH solution.	Test on watch-glass.
$\begin{array}{c} \text{NH} \text{---} \text{CO} \\   \quad   \\ \text{CO} \quad \text{CCH}_3 \\   \quad    \\ \text{NH} \text{---} \text{CH} \\ \text{(Thymin).} \end{array}$	Intense red color, which disappears on diluting with water.	Light red which fades rapidly.	Intense red color.
$\begin{array}{c} \text{CH}_3\text{N} \text{---} \text{CO} \\   \quad   \\ \text{CO} \quad \text{CCH}_3 \\   \quad    \\ \text{NH} \text{---} \text{CH} \\ \text{(1-Methylthymin.)} \end{array}$	Intense red color, which disappears on diluting with water.	No color.*	Intense red color.
$\begin{array}{c} \text{NH} \text{---} \text{CO} \\   \quad   \\ \text{CO} \quad \text{CCH}_3 \\   \quad    \\ \text{CH}_3\text{N} \text{---} \text{CH} \\ \text{(3-Methylthymin.)} \end{array}$	No color.	No color.	No color.
$\begin{array}{c} \text{CH}_3\text{N} \text{---} \text{CO} \\   \quad   \\ \text{CO} \quad \text{CCH}_3 \\   \quad    \\ \text{CH}_3\text{N} \text{---} \text{CH} \\ \text{(1, 3-Dimethylthymin.)} \end{array}$	No color.	No color.	No color.

\*Note—The statement—no color—indicates that no red color was formed.

TABLE II.

Pyrimidins.	0.5 cc. 10 per cent NaOH solution.	2.0 cc. $\frac{N}{10}$ NaOH solution.	Test on watch-glass.
$  \begin{array}{c}  \text{NH} \text{---} \text{CO} \\    \qquad   \\  \text{CO} \qquad \text{CH} \\    \qquad    \\  \text{NH} \text{---} \text{CH} \\  \text{(Uracil.)}  \end{array}  $	Red color but not as intense as with thymine.	Red color which developed slowly. Color permanent	Red color.
$  \begin{array}{c}  \text{CH}_3\text{N} \text{---} \text{CO} \\    \qquad   \\  \text{CO} \qquad \text{CH} \\    \qquad    \\  \text{NH} \text{---} \text{CH} \\  \text{(1-Methyluracil).}  \end{array}  $	Red color.	Red color.	Red color.
$  \begin{array}{c}  \text{NH} \text{---} \text{CO} \\    \qquad   \\  \text{CO} \qquad \text{CH} \\    \qquad    \\  \text{CH}_3\text{N} \text{---} \text{CH} \\  \text{(3-Methyluracil)}  \end{array}  $			
$  \begin{array}{c}  \text{CH}_3\text{N} \text{---} \text{CO} \\    \qquad   \\  \text{CO} \qquad \text{CH} \\    \qquad    \\  \text{CH}_3\text{N} \text{---} \text{CH} \\  \text{(1, 3-Dimethyluracil)}  \end{array}  $	No color.	No color.	No color.

TABLE III.

Pyrimidins.	0.5 cc. 10 per cent NaOH solution.	2.0 cc. $\frac{N}{10}$ NaOH solution.	Test on watch-glass.
$  \begin{array}{c}  \text{NH} \text{---} \text{CO} \\    \qquad   \\  \text{CO} \qquad \text{CBr} \\    \qquad    \\  \text{NH} \text{---} \text{CH} \\  \text{(5-Bromuracil.)}  \end{array}  $	Red color.	Faint red color which develops slowly.	Faint red color.
$  \begin{array}{c}  \text{CH}_3\text{N} \text{---} \text{CO} \\    \qquad   \\  \text{CO} \qquad \text{CBr} \\    \qquad    \\  \text{NH} \text{---} \text{CH} \\  \text{(1-Methyl-5-bromuracil.)}  \end{array}  $	Red color.		
$  \begin{array}{c}  \text{NH} \text{---} \text{CO} \\    \qquad   \\  \text{CO} \qquad \text{CBr} \\    \qquad    \\  \text{CH}_3\text{N} \text{---} \text{CH} \\  \text{(3-Methyl-5-bromuracil.)}  \end{array}  $	No color.	No color.	No color.
$  \begin{array}{c}  \text{CH}_3\text{N} \text{---} \text{CO} \\    \qquad   \\  \text{CO} \qquad \text{CBr} \\    \qquad    \\  \text{CH}_3\text{N} \text{---} \text{CH} \\  \text{(1, 3-Dimethyl-5-bromuracil.)}  \end{array}  $	No color.	No color.	No color.

TABLE IV.

Pyrimidins.	0.5 cc. 10 per cent NaOH solution.	2.0 cc. $\frac{N}{10}$ NaOH solution.	Test on watch-glass.
$  \begin{array}{c}  \text{N} = \text{C.NH}_2 \\    \quad   \\  \text{CO} \quad \text{CH.H}_2\text{O} \\    \quad    \\  \text{NH} - \text{CH} \\  \text{(Cytosin.)}  \end{array}  $	Red color which developed slowly.	Faint red.	Red color like uracil.
$  \begin{array}{c}  \text{N} = \text{CNH}_2 \\    \quad   \\  \text{CO} \quad \text{CH} \\    \quad    \\  \text{CH}_3\text{N} - \text{CH} \\  \text{(3-Methyl-cytosin.)}  \end{array}  $	No color.	No color.	No color.
$  \begin{array}{c}  \text{N} = \text{CN} \begin{array}{l} \nearrow \text{CH}_3 \\ \searrow \text{C}_6\text{H}_5 \end{array} \\    \quad   \\  \text{CO} \quad \text{CH} \\    \quad    \\  \text{NH} - \text{CH}  \end{array}  $	Red color.	No color.	Red color.
$  \begin{array}{c}  \text{N} = \text{CN} \begin{array}{l} \nearrow \text{CH}_3 \\ \searrow \text{C}_6\text{H}_5 \end{array} \\    \quad   \\  \text{CO} \quad \text{CH} \\    \quad    \\  \text{CH}_3\text{N} - \text{CH}  \end{array}  $	No color.	No color.	No color.

TABLE V.

Pyrimidins.	0.5 cc. 10 per cent NaOH solution.	2.0 cc. $\frac{N}{10}$ NaOH solution.	Test on watch-glass.
$  \begin{array}{c}  \text{N} = \text{CNH}_2 \\    \quad   \\  \text{CO} \quad \text{CCH}_3 \\    \quad    \\  \text{NH} - \text{CH} \\  \text{(5-Methyl-cytosin)}  \end{array}  $	Intense red color.	Faint red which faded.	Intense red.
$  \begin{array}{c}  \text{N} = \text{CNH}_2 \\    \quad   \\  \text{CO} \quad \text{CCH}_3 \\    \quad    \\  \text{CH}_3\text{N} - \text{CH} \\  \text{(3, 5-Dimethyl-cytosin.)}  \end{array}  $	No color.	No color.	No color.

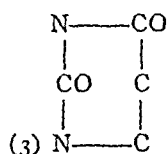
## SUMMARY.

(1) Thymin, uracil and cytosin react with diazobenzene sulfonic acid, in presence of alkali, giving red colored solutions.

(2) The color is given by thymin with greater intensity than by uracil and cytosin.

(3) Substitution in position 3 of the pyrimidin ring prevents the formation of a red color.

(4) Accepting the statement of Burian,<sup>1</sup> that nucleic acids do not react with diazobenzene sulfonic acid, the foregoing observations seem to indicate that thymin and probably uracil and cytosin as well are linked in nucleic acids at position 3.



(5) Whether the pyrimidins are linked to phosphorus, a carbohydrate complex or otherwise must be decided by further study.



# FURTHER STUDIES OF THE MODE OF OXIDATION OF PHENYL DERIVATIVES OF FATTY ACIDS IN THE ANIMAL ORGANISM.

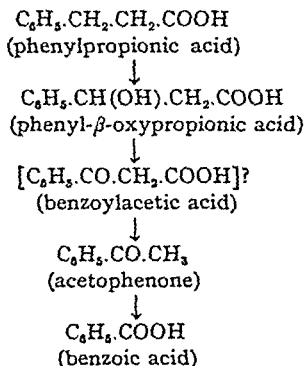
(PHENYLBUTYRIC ACID, PHENYL- $\beta$ -OXYBUTYRIC ACID, PHENYLACE-  
TONE, PHENYLISOCROTONIC ACID, PHENYL- $\beta,\gamma$ -DIOXY-  
BUTYRIC ACID.)

By H. D. DAKIN.

(From the Laboratory of Dr. C. A. Herter, New York.)

(Received for publication, August 12, 1908.)

In previous papers<sup>1</sup> it was shown that phenylpropionic acid, at least in part, underwent oxidation in the animal organism in accordance with the following scheme:



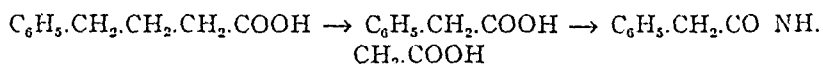
Benzoylactic acid was not detected but its formation was inferred from the production of acetophenone, into which it readily passes through loss of carbon dioxide. At the same time it was thought probable that some benzoic acid was formed without passing through the stage of acetophenone.

The close analogy between the apparent mode of catabolism of phenylpropionic acid and that of butyric acid made it desirable

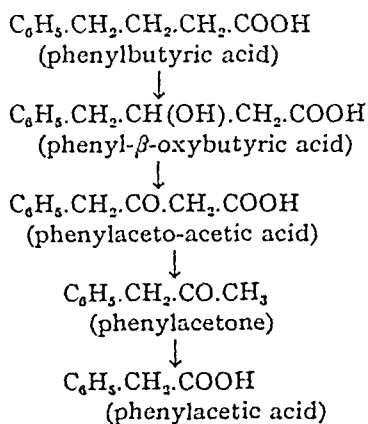
<sup>1</sup> This *Journal*, iv, p. 419, 1908; *Beitr. z. chem. Physiol. u. Pathol.*, xi, p. 404.

to extend the investigation to other aromatic derivatives of the fatty acids in the hope of obtaining further insight into the processes of tissue oxidation of the fatty acids of physiological importance. The present communication deals with the fate in the body of a number of derivatives of phenylbutyric acid.

The fate of phenylbutyric acid in the organism has already been investigated by F. Knoop<sup>1</sup> who administered it by mouth to a dog and observed the subsequent excretion of phenaceturic acid in the urine. This result was interpreted in the light of Knoop's well-known hypothesis of  $\beta$ -oxidation and indeed was one of the most important facts upon which his theory was based. The conversion of phenylbutyric acid into phenaceturic acid involves the intermediate formation of phenylacetic acid which is then paired with glycocoll:



It is clearly desirable to ascertain the intermediate steps in the conversion of phenylbutyric acid into phenylacetic acid. So far as I am aware no picture of the mechanism of the reaction has hitherto been put forward. In order to elucidate this mechanism it was natural to inquire first of all whether the catabolism of phenylbutyric acid did not follow upon the same line as that of phenylpropionic acid. Judging by analogy one might expect the change to be as follows:



<sup>1</sup> *Beitr. z. chem. Physiol. u. Pathol.*, vi, p. 155, 1904.

The occurrence of the first step in this series of changes, namely, the formation of phenyl- $\beta$ -oxybutyric acid, is highly probable, as is shown by the following facts:

(1) A small quantity of a laevorotatory substance giving the reactions of phenyl- $\beta$ -oxybutyric acid was isolated from the urine of dogs that had received subcutaneous injections of sodium phenylbutyrate in fairly large doses. The  $\beta$ -oxy-acid could not, however, be isolated in a state of purity.

(2) Phenyl- $\beta$ -oxybutyric acid injected in the form of its sodium salt was excreted in the form of phenaceturic acid, i. e., the same end-product as phenylbutyric acid itself yields.

There was little hope of isolating the substance corresponding to the second hypothetical step in the reaction, namely, phenyl-aceto-acetic acid, for this body has not yet been synthesized and would doubtless be unstable. It is improbable, however, that it was present in the urines of the dogs which had received injections of sodium phenylbutyrate, because in this case phenylacetone would have been found in the distillates, for a  $\beta$ -ketonic acid of the type of phenylaceto-acetic acid doubtless would lose carbon dioxide on boiling, with formation of the corresponding ketone.

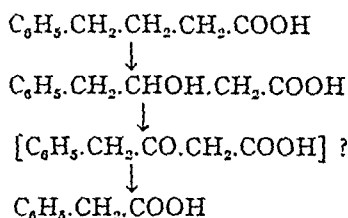
The third possible stage in the catabolism of phenylbutyric acid involving the formation of phenylacetone, corresponding to the intermediate production of acetophenone from phenyl-propionic acid, was definitely excluded on the basis of the following experimental results:

(1) No trace of phenylacetone could be detected in the urines of animals which had received injections of considerable quantities of phenylbutyric acid.

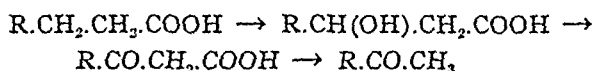
(2) Phenylacetone cannot be an intermediate stage in the catabolism of phenylbutyric acid for when administered to dogs it results in the excretion of *hippuric acid*. Phenylbutyric acid under similar conditions gives *phenaceturic acid*.

To sum up: Evidence has been obtained that phenylbutyric acid when oxidized in the body passes through the stage of phenyl- $\beta$ -oxybutyric acid. No evidence could be obtained of the formation of phenylaceto-acetic acid and it could not be detected in the urine. The possibility of its formation as an intermediate product is not, however, excluded. Phenylacetone is certainly *not* a product of the catabolism of phenylbutyric acid.

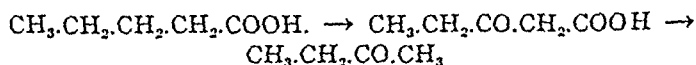
It is clear, therefore, that the modes of catabolism of phenylpropionic acid and of phenylbutyric acid, though similar as regards the primary formation of a  $\beta$ -oxy-acid, differ in that ketone formation takes place in the case of phenylpropionic acid but not in that of phenylbutyric acid. The catabolism of phenylbutyric acid is therefore to be represented as follows:



The mechanism of the catabolism of phenylbutyric acid appears to be of interest from several points of view. In the first place it furnishes an additional example of the primary oxidation of the hydrogen attached to the  $\beta$ -carbon atom of a phenyl-fatty acid with formation of a laevorotatory  $\beta$ -oxy-acid. In the second place it indicates the possibility, which has been insisted on in previous papers, of a  $\beta$ -oxy or  $\beta$ -ketonic acid undergoing oxidation without intermediate ketone formation.<sup>1</sup> *Indeed it would appear as if ketone formation were restricted to the simplest members of the fatty acids and phenyl-fatty acids, theoretically capable of ketone formation, namely, butyric acid and phenylpropionic acid:*



This hypothesis furnishes an explanation of why under conditions such as diabetes in which large quantities of ketones are excreted only acetone has been detected. If the above scheme represented a perfectly general type of reaction, it would be hard to explain why in diabetes the catabolism of acids such as caproic and valeric do not give rise to the excretion of propylmethyl ketone or ethylmethyl ketone respectively:



<sup>1</sup> Unpublished experiments upon the mode of catabolism of phenylvaleric and phenyl- $\beta$ -oxyvaleric acid indicate that the substances resemble phenylbutyric acid in this respect.

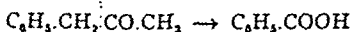
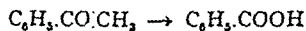
It is at present impossible to decide what rôle, if any, the  $\beta$ -ketonic acids, other than aceto-acetic acid play in intermediary metabolism. Apart from aceto-acetic acid and benzoylacetic acid few if any of these acids of a type which might be formed in metabolism, have been prepared. It may be that they are extremely unstable or even incapable of more than momentary existence. It is well, however, to bear in mind that not only are  $\beta$ -ketonic acids capable of undergoing hydrolysis according to the well-known scheme



a reaction which fits in well with the progressive degradation of long-chain fatty acids by the loss of two carbon atoms at a time, but also that they are extremely easily oxidizable substances.<sup>1</sup>

If the normal course of metabolism of a straight-chain fatty acid other than butyric acid and phenylpropionic acid, proceeds through the  $\beta$ -oxy-acids and possibly the  $\beta$ -ketonic acids, but does not involve ketone formation, is it not probable that the catabolism of butyric and phenylpropionic acid in part does not involve the intermediate formation of acetone and acetophenone respectively? There is a certain amount of physiological evidence in support of this view, and so far as I know, there is none opposed to it. The results of oxidation experiments *in vitro* furnish complete chemical analogy for these reactions.<sup>2</sup>

The excretion of hippuric acid following the administration of phenylacetone is of interest especially when it is considered that its lower homologue, acetophenone, also yields hippuric acid. These changes are similar to the action of ordinary oxidizing agents (including hydrogen peroxide) which oxidize both ketones to benzoic acid. It will probably be found that most aromatic methyl ketones primarily undergo oxidation in the body, so as to yield acids with two less carbon atoms, except in the case of acetophenone, in which the carbonyl group is directly attached to the nucleus:



<sup>1</sup> A communication upon this subject will be made shortly.

<sup>2</sup> This *Journal*, iv, p. 77.



## EXPERIMENTAL PART.

*Preparation of phenylbutyric acid.* Phenylbutyric acid has been synthesized by several methods, the most direct of these being the reduction of phenylisocrotonic acid with sodium amalgam as described by Fittig and Jayne.<sup>1</sup> The reduction, however, is not very easily carried out as was found by Jayne and by Knoop and also in experiments of my own. Kipping and Hill,<sup>2</sup> on the other hand, apparently had no difficulty in effecting the reduction at the ordinary temperature. On the whole, it was found more advantageous to employ the following method, mainly, based on the investigations of Fittig and his pupils. Phenylparaconic acid, prepared by means of Perkin's reaction from benzaldehyde, sodium succinate and acetic anhydride,<sup>3</sup> is distilled *in vacuo*. The distillate, consisting mainly of phenylisocrotonic acid with a little phenylbutyrolactone, is boiled with twenty-five parts of hydrochloric acid (1 part concentrated acid, 3 parts water by volume) under a reflux condenser, for six hours. The result of this procedure is to convert about 65 per cent of the phenylisocrotonic acid into phenylbutyrolactone.<sup>4</sup> The acid is separated from the lactone by adding sodium carbonate till faintly alkaline to the ethereal extract containing the two substances.<sup>5</sup> The acid remaining in the aqueous layer is again treated with hydrochloric acid. In this way an 80 per cent yield of phenylbutyrolactone is readily obtained. The phenylbutyrolactone is reduced to phenylbutyric acid according to Shield's method<sup>6</sup> by boiling it with ten parts of hydriodic acid (b. p. 127°) and 1.5 parts of red phosphorus for ten hours. The phenylbutyric acid is obtained by ether extraction after previous dilution with water and after removing the ether by evaporation readily crystallizes. The method gives an excellent yield. Twenty-five grams of phenylparaconic acid gave on the average

<sup>1</sup> *Ann. d. Chem.*, ccxvi, p. 108.

<sup>2</sup> *Trans. Chem. Soc.*, lxxv, p. 147.

<sup>3</sup> *Ann. d. Chem.*, ccxvi, p. 100.

<sup>4</sup> Fittig and Hadorff: *Ibid.*, cccxxxiv, p. 117.

<sup>5</sup> This method of converting phenylisocrotonic acid into phenylbutyrolactone was found far superior to that of Erdmann who employed 33 per cent sulphuric acid. *Ibid.*, ccxxviii, p. 178.

<sup>6</sup> *Ibid.*, cclxxxviii, p. 207.





*Fate of phenylacetone in the organism.* Phenylacetone (Kahlbaum) was injected subcutaneously into dogs in dilute alcoholic solution in doses varying from 3 to 4 grams. The absorption of the ketone appeared to be slow and in one case comparatively little hippuric acid was obtained (0.3 gram). In all the other cases an abundant yield of hippuric acid was obtained—in one case as much as 3.0 grams being recovered. A small amount of unchanged ketone was excreted in the urine. The hippuric acid after recrystallization, melted sharply at  $187^{\circ}$ .

*Fate of phenylisocrotonic acid in the organism.* Phenylisocrotonic acid was prepared by distilling phenylparaconic acid *in vacuo* and crystallizing the distillate from carbon bisulphide. Two grams of the acid were dissolved in alcohol and almost neutralized with caustic soda. The solution was injected subcutaneously into a cat (two kilos). The urine was collected for three days. It contained a trace of acetone but no aromatic ketone. The urine was concentrated to about 100 cc., acidified with phosphoric acid and extracted in a continuous extractor with ethyl acetate. After purifying the ethyl acetate extract by steam distillation and by boiling the aqueous solution with charcoal, 0.65 gram of pure phenaceturic acid, melting at  $142^{\circ}$ , was obtained. No hippuric acid could be detected.

*Fate of phenyl- $\beta$ , $\gamma$ -dioxybutyric acid.* This acid was prepared by Fittig and Obermüller's method<sup>1</sup> by oxidizing phenylisocrotonic acid in dilute alkaline solution at  $0^{\circ}$  with dilute potassium permanganate. The product obtained was a mixture of the free acid and lactone and was converted into the sodium salt by boiling with excess of caustic soda, neutralizing with acetic acid and injecting the dilute solution subcutaneously into a cat of about 2.5 kilos weight. One and a quarter grams of the acid in the form of sodium salt gave about 0.45 gram of pure crystalline hippuric acid (m.p.  $185^{\circ}$  to  $187^{\circ}$ ) and about 0.2 gram of phenyl- $\beta$ -oxybutyrolactone. The lactone was separated from the hippuric acid as follows: The urine was concentrated, acidified and extracted with ether in the usual way. The ethereal residue was distilled in steam for a very short time only, then decolorized with charcoal and concentrated to about 5 cc. The

<sup>1</sup> Liebig's *Ann. d. Chem.*, cclxviii, p. 44.

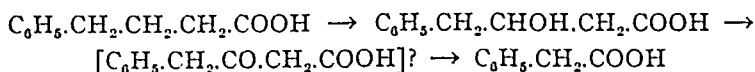
solution was then made just alkaline with sodium carbonate solution and extracted with ether to remove the lactone. The alkaline residue was acidified and again repeatedly extracted with ether. On evaporation, hippuric acid crystals were obtained in abundance. The aqueous solution of the ethereal extract was optically inactive and no positive indication of the presence of mandelic acid could be obtained.

#### SUMMARY.

The subcutaneous injection of phenylbutyric acid in the form of its sodium salt in aqueous solution results in the excretion of phenaceturic acid as found by Knoop. In addition a small quantity of a lævorotatory acid possessing the properties of phenyl- $\beta$ -oxybutyric acid was excreted. No phenylacetone was excreted and phenylaceto-acetic acid could not be detected.

Phenyl- $\beta$ -oxybutyric acid administered under similar conditions results in the excretion of phenaceturic acid. No phenylacetone could be detected. A part of the oxy-acid is apparently excreted unchanged and is lævorotatory.

Administration of phenylacetone results in the excretion of hippuric acid, no phenaceturic acid being formed. Phenylacetone cannot therefore be an intermediate product of the catabolism of phenylbutyric acid. The probable mode of oxidation of phenylbutyric acid in the body may be represented as follows:



The phenylacetic acid is excreted in the form of phenaceturic acid.

A comparison is made between the mode of oxidation of phenylbutyric acid and that of phenylpropionic acid. The first step in the catabolism of both acids apparently consists in the formation of a  $\beta$ -oxy-acid but in the case of phenylbutyric acid there is no formation of the corresponding ketone as a product of further oxidation. The intermediate formation of ketones observed in the catabolism of butyric and phenylpropionic acids is probably confined to these two acids and is not a general reaction.

These results are in harmony with the view that in normal metabolism probably only part of the butyric acid and phenylpropionic acid undergoing oxidation passes through the stages of acetone and acetophenone respectively.

Phenylisocrotonic acid administered subcutaneously to cats in the form of its sodium salt is excreted in the form of phenaceturic acid.

Phenyl- $\beta,\gamma$ -dioxymybutyric acid administered to cats in the form of its sodium salt resulted in the excretion of hippuric acid together with a little phenyl- $\beta$ -oxymybutyrolactone. No indications could be obtained of the formation of mandelic acid. Phenyl-dioxymybutyric acid therefore does not undergo  $\beta$ -oxidation but oxidation takes place at the  $\gamma$ -carbon atom. Phenyl-dioxymybutyric acid is not a product of the catabolism of phenylbutyric acid.

Phenylacetone is readily identified by conversion into its para-nitrophenylhydrazone which crystallizes from alcohol or pyridin in sparingly soluble rosettes of platelets melting at  $145^{\circ}$  to  $145.5^{\circ}$ .

*Note added during proof correction.* The investigation of the fate of phenylvaleric and phenyl- $\beta$ -oxyvaleric acid has shown that while the end product of catabolism in both cases is hippuric acid, *cinnamylglycocol*,  $C_6H_5.CH:CH.CO.NH.CH_2.COOH$  m. p.,  $193^{\circ}$ , is an intermediate product of their catabolism. This substance is also produced in the oxidation of phenylpropionic acid in the animal body. These observations throw considerable light upon the mechanism of fatty acid metabolism and will form the subject of a separate communication.



## HYDROLYSIS OF VICILIN FROM THE PEA (*Pisum Sativum*).<sup>1</sup>

By THOMAS B. OSBORNE AND FREDERICK W. HEYL.

(From the Laboratory of the Connecticut Agricultural Experiment Station.)

(Received for publication, July 23, 1908.)

Vicilin is a globulin which occurs in the seeds of the pea, horse-bean and lentil. Its isolation, properties and composition have been given in a series of papers from this laboratory.<sup>2</sup> It differs from legumin in containing a little more carbon, a little less nitrogen and less than half as much sulphur, and also in being soluble in much more dilute saline solutions.

Vicilin presents a special interest because it contains the smallest proportion of sulphur yet found in any protein. A large number of preparations made and analyzed in this laboratory, contained from 0.10 to 0.20 per cent of total sulphur. This difference is not analytical, for the extreme figures were confirmed in several cases by very closely agreeing duplicate determinations. The sulphur content of vicilin as heretofore prepared, is not constant and deserves further careful study, for it would appear that either the sulphur-containing complex of this protein is very easily split off, or that the preparations were mixtures of protein free from sulphur and protein containing sulphur. Owing to the great solubility of vicilin, it has not yet been subjected to sufficiently extensive fractionation to determine this latter point. That it is a mixture of sulphur-free protein and legumin is not probable, for the differences in sulphur content are not accompanied by the differences in carbon and nitrogen which would be expected if this were the case.

<sup>1</sup> The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

<sup>2</sup> Osborne and Campbell: *Journ. Amer. Chem. Soc.*, xx, pp. 348, 362, 393, 410, 1898.

It is probable that vicilin is a distinct protein or mixture of proteins and not an altered product of legumin, for the seeds of the vetch which contain legumin yield no vicilin. The resemblance in properties and composition between vicilin and legumin makes it desirable to know whether a more positive difference can be found in the nature and proportion of the amino-acids which each yields when hydrolyzed.

The results of the hydrolysis are given in the following table together with those previously obtained for pea legumin.

	Vicilin. per cent.	Legumin. per cent.
Glycocoll.....	0.00	0.38
Alanine.....	0.50	2.08
Valine.....	0.15	?
Leucine.....	9.38	8.00
Proline.....	4.06	3.22
Phenylalanine.....	3.82	3.75
Aspartic acid.....	5.30	5.30
Glutaminic acid.....	21.34	16.97
Serine.....	?	0.53
Cystine.....	not det.	not det.
Oxyproline.....	"	"
Tyrosine .....	2.38	1.55
Arginine.....	8.91	11.71
Histidine.....	2.17	1.69
Lysine.....	5.40	4.98
Ammonia.....	2.03*	2.05*
Tryptophane.....	present	present
Total.....	65.44	62.22

\* Osborne and Harris: *Journ. Amer. Chem. Soc.*, xxv, p. 323, 1903.

The results of these hydrolyses are very similar, the most marked difference being that vicilin yielded no glycocoll. Less alanine and arginine and more glutaminic acid were also found than in legumin. Although special effort was made to isolate glycocoll from vicilin no trace of it was found, and it is probable that vicilin in fact yields none. It is to be noted however that glycocoll can be missed when present in small amount. We have recently made two hydrolyses on the same preparation of legumin from the vetch, in one of which a small quantity of glycocoll was found while from the other none whatever was obtained.

This difference we attribute to the fact that the esters of the latter hydrolysis, owing to an accident to the liquid air apparatus, had to be kept in ether for some weeks before distilling, during which time the ester of glycoll presumably underwent change. That this occurred in the hydrolysis of vicilin is improbable, for the esters were distilled soon after they were made. The difference in the proportion of alanine in legumin and vicilin is relatively great, and it is probable that an actual difference exists between these proteins in this respect. The vicilin used for this hydrolysis was prepared in the way described in a former paper<sup>1</sup> from this laboratory.

#### HYDROLYSIS OF VICILIN.

A quantity of vicilin was taken for hydrolysis which weighed 552.8 grams, ash and moisture-free. This was treated with 1200 cc. of hydrochloric acid of 1.10 specific gravity. The vicilin was brought into solution by heating on the water-bath for about three hours, and hydrolysis was completed by boiling in an oil-bath for twenty hours.

The solution was then concentrated under reduced pressure, saturated at 0° with hydrochloric acid gas, and allowed to stand on ice for four days. The glutaminic acid hydrochloride which separated weighed 127.77 grams, which after deducting the amount of ammonium chloride which it contained, is equivalent to 102.38 grams of glutaminic acid, or 18.52 per cent of the protein.

The glutaminic acid hydrochloride, after one recrystallization, was analyzed, with the following result.

Chlorine, 0.2830 gm. subst. gave 0.2232 gm. AgCl.

Nitrogen, 0.3076 gm. subst. required 16.55 cc.  $\frac{1}{10}$  N.HCl.

	Calculated for $C_5H_9O_4N.HCl$ :	Found:
Cl.....	19.32	19.49
N.....	7.63	7.53

The free glutaminic acid decomposed at 202° to 203°.

<sup>1</sup> Osborne and Harris: This *Journal*, iii, p. 213, 1907.

Carbon and hydrogen, 0.2821 gm. subst. gave 0.4234 gm.  $\text{CO}_2$  and 0.1604 gm.  $\text{H}_2\text{O}$ .

Calculated for  $\text{C}_5\text{H}_9\text{O}_4\text{N}$ .....C = 40.81; H = 6.12 per cent.

Found.....C = 40.94; H = 6.31 "

The filtrate from the glutaminic acid hydrochloride was concentrated under greatly reduced pressure and esterified with alcohol and dry hydrochloric acid gas, as directed by Emil Fischer. The esters were liberated, shaken out and dried in the usual manner, and the aqueous layer freed from inorganic salts and again esterified.

A third esterification was also carried out, but only a little more ether soluble ester was obtained.

The ether was distilled off on the boiling water-bath, and the residual esters distilled under diminished pressure into the following fractions.

Fraction.	Temp. of bath up to	Pressure, mm.	Weight, grams.
I.....	65°	12.00	14.83
II.....	100°	10.00	49.33
IIIa.....	100°	1.00	76.27
IIIb.....	110°	1.00	13.57
IV.....	147°	0.85	56.92
V.....	158°	0.75	33.85
VI.....	200°	0.70	28.75
Total.....			273.52

The undistilled residue weighed 76 grams.

Between 65° and 87° at 10 mm. nothing was distilled.

*Fraction I.* From this fraction no glyocoll could be separated as the hydrochloride of the ethyl ester. The ether which had been distilled from the esters also yielded a negative result. The esters were saponified and after removing chlorine, added to the corresponding part of Fraction II.

*Fraction II.* This fraction was saponified by boiling with six volumes of water for seven hours. The solution of the amino-acids was evaporated to dryness under reduced pressure and the proline extracted by boiling with absolute alcohol.

The amino-acids, insoluble in alcohol, were taken up in water and the greater part of the leucine was separated by fractional crystallization. The leucine weighed 13.82 grams.



Owing to the persistence of the characteristic leucine-valine mixture, the filtrate from the leucine was racemized by heating with an excess of baryta in an autoclave for twenty-four hours at  $175^{\circ}$ .

After removing the barium quantitatively with sulphuric acid, the amino-acids were fractionally crystallized, and 0.84 gram of substance obtained, which had the composition of amino-valerianic acid.

*Carbon and hydrogen*, I, 0.1255 gm. subst. gave 0.2349 gm.  $\text{CO}_2$  and 0.1107 gm.  $\text{H}_2\text{O}$ . II, 0.1612 gm. subst. gave 0.3012 gm.  $\text{CO}_2$  and 0.1332 gm.  $\text{H}_2\text{O}$ .

Calculated for  $\text{C}_5\text{H}_{11}\text{O}_2\text{N} \dots \text{C} = 51.28$ ;  $\text{H} = 9.40$  per cent.

Found.....I,  $\text{C} = 51.04$ ;  $\text{H} = 9.80$  "

II,  $\text{C} = 50.95$ ;  $\text{H} = 9.18$  "

The filtrate from the valine yielded 2.80 grams of alanine. The alanine crystallized from dilute alcohol in bunches of needles and when recrystallized from water, was obtained in characteristic prisms, which decomposed above  $290^{\circ}$ .

*Carbon and hydrogen*, 0.1445 gm. subst. gave 0.2165 gm.  $\text{CO}_2$  and 0.1040 gm.  $\text{H}_2\text{O}$ .

Calculated for  $\text{C}_3\text{H}_7\text{O}_2\text{N} \dots \text{C} = 40.45$ ;  $\text{H} = 7.87$  per cent.

Found..... $\text{C} = 40.86$ ;  $\text{H} = 7.99$  "

The more soluble part of this fraction was examined for glycoll before it was racemized, but none was found.

*Fraction IIIa.* This fraction was boiled, with five volumes of water for seven hours with return condenser. The leucine which had separated was filtered off, the filtrate evaporated to dryness under diminished pressure, and the proline extracted with boiling absolute alcohol.

The amino-acids, insoluble in alcohol, yielded 35.59 grams of leucine which decomposed at  $298^{\circ}\text{C}$ .

*Carbon and hydrogen*, 0.1256 gm. subst. gave 0.2533 gm.  $\text{CO}_2$  and 0.1130 gm.  $\text{H}_2\text{O}$ .

Calculated for  $\text{C}_6\text{H}_{13}\text{O}_2\text{N} \dots \text{C} = 54.96$ ;  $\text{H} = 9.92$  per cent

Found..... $\text{C} = 55.01$ ;  $\text{H} = 10.00$  "

The alcoholic proline solutions obtained from Fractions II and III were united and evaporated to dryness under reduced pressure.

The residue was taken up in boiling absolute alcohol, and this process repeated several times until the residue was completely soluble in cold absolute alcohol. This solution yielded 27.96 grams of lævoproline copper, dried at  $110^{\circ}$ , and 0.60 gram racemic proline copper, equivalent to 22.48 grams proline, or 4.06 per cent.

The racemic salt was analyzed as follows.

*Water*, 0.1429 gm. subst. air dry lost 0.0151 gm.  $H_2O$  at  $110^{\circ}$ .

*Copper*, 0.1065 gm. subst. air dry gave 0.0259 gm.  $CuO$ .

Calculated for  $C_{10}H_{16}O_4N_2Cu \cdot 2H_2O \cdot H_2O = 10.99$  p. c.;  $Cu = 19.40$  p. c.  
 Found.....  $H_2O = 10.57$  "  $Cu = 19.43$

The amorphous lævoproline copper was converted into the phenylhydantoine, which after one recrystallization from a large volume of water, melted at  $143^{\circ}$ , and had the following composition.

*Carbon and hydrogen*, 0.1294 gm. subst. gave 0.3170 gm.  $CO_2$  and 0.0667 gm.  $H_2O$ .

Calculated for  $C_{12}H_{12}O_2N_2$ .....  $C = 66.67$ ;  $= H 5.57$  per cent.  
 Found.....  $C = 66.81$ ;  $= H 5.72$  "

*Fraction IIIb.* This fraction was shaken out with an equal volume of ether, and the ether layer was washed with water in the usual manner. From the ether layer there was obtained 3.09 grams of leucine hydrochloride which when converted into leucine gave the following analysis.

*Carbon and hydrogen*, 0.1326 gm. subst. gave 0.2655 gm.  $CO_2$  and 0.1158 gm.  $H_2O$ .

Calculated for  $C_6H_{13}O_2N$ .....  $C = 54.96$ ;  $H = 9.92$  per cent.  
 Found.....  $C = 54.60$ ;  $H = 9.70$  "

The aqueous layer was added to the corresponding solution from Fraction IV.

*Fraction IV.* By a similar treatment, this fraction yielded 7.96 grams of phenylalanine hydrochloride; 20.05 grams of aspartic acid as the barium salt; and 1.46 grams of copper aspartate.

The aspartic acid reddened but did not decompose at  $300^{\circ}$ .

After one crystallization from water, the following analysis was obtained.

*Carbon and hydrogen*, 0.2185 gm. subst. gave 0.2914 gm.  $\text{CO}_2$  and 0.1100 gm.  $\text{H}_2\text{O}$ .

Calculated for  $\text{C}_4\text{H}_7\text{O}_4\text{N}$ .....C = 36.09; H = 5.26 per cent.

Found.....C = 36.36; H = 5.59 "

*Fraction V.* This fraction yielded 9.55 grams of very pure phenylalanine hydrochloride. The free acid decomposed at  $270^\circ$ .

*Carbon and hydrogen*, 0.1515 gm. subst. gave 0.3628 gm.  $\text{CO}_2$  and 0.0936 gm.  $\text{H}_2\text{O}$ .

Calculated for  $\text{C}_9\text{H}_{11}\text{O}_2\text{N}$ .....C = 65.45; H = 6.67 per cent.

Found.....C = 65.31; H = 6.86 "

There were further obtained from the aqueous layer, in the usual manner, 4.85 grams of aspartic acid, as the barium salt. The aspartic acid reddened at  $300^\circ$ , but did not decompose. The filtrate from the barium aspartate was freed from barium, concentrated to a small volume under diminished pressure, and saturated with hydrochloric acid gas at  $0^\circ$ . There separated 3.87 grams of glutaminic acid hydrochloride.

After recrystallization, it decomposed at  $198^\circ$ , and had the following composition.

*Chlorine*, 0.1382 gm. subst. gave 0.1069 gm.  $\text{AgCl}$ .

Calculated for  $\text{C}_5\text{H}_9\text{O}_4\text{N} \cdot \text{HCl}$ .....Cl = 19.32 per cent.

Found.....Cl = 19.12 "

The filtrate from the glutaminic acid hydrochloride was freed from chlorine and 6.79 grams of air dry copper aspartate were obtained from it.

*Copper*, 0.1552 gm. subst. air dry gave 0.0451 gm.  $\text{CuO}$ .

*Nitrogen*, 0.3484 gm. subst. air dry required 13.1 cc.  $\frac{\text{N}}{\text{V}_0} = \text{HCl}$ .

Calculated for  $\text{C}_4\text{H}_5\text{O}_4\text{NCu} \cdot 4\frac{1}{2}\text{H}_2\text{O}$  .. Cu = 23.07; N = 5.08 per cent.

Found.....Cu = 23.21; N = 5.26 "

*Fraction VI.* From this fraction there were obtained 8.46 grams of phenylalanine hydrochloride, 2.52 grams of glutaminic acid as the barium salt, 5.95 grams of glutaminic acid hydrochloride, and 1.09 grams of air dry copper aspartate. The glutaminic acid from the barium salt gave Analysis I and that from the hydrochloride gave Analysis II.

Carbon and hydrogen, I, 0.3027 gm. subst. gave 0.4514 gm.  $\text{CO}_2$  and 0.1622 gm.  $\text{H}_2\text{O}$ . II, 0.2445 gm. subst. gave 0.3685 gm.  $\text{CO}_2$  and 0.1419 gm.  $\text{H}_2\text{O}$ .

Calculated for  $\text{C}_5\text{H}_9\text{O}_4\text{N}$ .....C = 40.81; H = 6.12 per cent.

Found.....I, C = 40.67; H = 5.95 "

II, C = 41.10; H = 6.44 "

### *Residue after Distillation.*

The residue which remained after the distillation of the esters yielded 4.4 grams of diketopiperazines, and 6.47 grams of glutamic acid hydrochloride. The free acid decomposed at  $202^\circ$  to  $203^\circ$  C.

Carbon and hydrogen, 0.1500 gm. subst. gave 0.2255 gm.  $\text{CO}_2$  and 0.0834 gm.  $\text{H}_2\text{O}$ .

Calculated for  $\text{C}_5\text{H}_9\text{O}_4\text{N}$ .....C = 40.81; H = 6.12 per cent.

Found.....C = 41.00; H = 6.17 "

### *Cystine.*

Owing to the very small proportion of sulphur in vicilin, no attempt was made to determine cystine.

### *Tyrosine.*

A quantity of vicilin equal to 36.57 grams ash and moisture-free was hydrolyzed by boiling with a mixture of 120 grams of sulphuric acid and 240 cc. of water for twenty-four hours in an oil-bath. After freeing the solution from sulphuric acid, it was concentrated to crystallization and cooled. The substance which separated was recrystallized from water and yielded 0.8708 gram of tyrosine, equal to 2.38 per cent.

Nitrogen, 0.1090 gm. subst. required 5.8 cc.  $\frac{N}{15}$  HCl.

Calculated for  $\text{C}_9\text{H}_{11}\text{O}_3\text{N}$ .....N = 7.73 per cent.

Found.....N = 7.45 "

The filtrate from the tyrosine was used for determination of the bases according to the method of Kossel and Patten.

### *Histidine.*

The solution of the histidine = 500 cc.

Nitrogen, 50 cc. sol. required 2.15 cc.  $\frac{5N}{7}$  HCl = 0.2150 gm. N in 500 cc. = 0.7934 gm. histidine = 2.17 per cent.

The histidine was identified as the dichloride.

*Chlorine*, 0.1377 gm. subst. gave 0.1718 gm. AgCl.

Calculated for  $C_6H_6O_2N_3$ .....Cl = 31.14 per cent.

Found.....Cl = 30.86 "

### *Arginine.*

The solution of the arginine = 1000 cc.

*Nitrogen*, 50 cc. sol. required 5.07 cc.  $\frac{5N}{7}$  HCl = 1.0140 gm. N in 1000 cc. = 3.1505 gm. arginine + 0.1102 gm. = 3.2607 gm. or 8.91 per cent.

The arginine was converted into the copper-nitrate double salt for identification.

*Copper*, 0.1713 gm. subst., air dry, gave 0.0235 gm. CuO.

Calculated for  $C_{12}H_{28}O_4N_8Cu(NO_3)_2 \cdot 3H_2O$ ... Cu = 10.79 per cent.

Found.....Cu = 10.96 "

### *Lysine.*

The lysine picrate weighed 5.0707 grams equal to 1.9740 grams lysine or 5.40 per cent. The lysine was identified as the picrate.

*Nitrogen*, 0.1355 gm. subst. required 18.25 cc.  $\frac{N}{10}$  HCl.

Calculated for  $C_6H_{14}O_2N_2 \cdot C_6H_3O_7N_3$ ..... N = 18.70 per cent.

Found..... N = 18.85 "



## HYDROLYSIS OF LEGUMELIN FROM THE PEA (*Pisum Sativum*).<sup>1</sup>

By THOMAS B. OSBORNE AND FREDERICK W. HEYL.

(From the Laboratory of the Connecticut Agricultural Experiment Station,  
New Haven, Conn.)

(Received for publication, July 23, 1908.)

Legumelin is a name that has been applied to the albumin which occurs in the seeds of a number of different leguminous plants. As the properties, composition and occurrence of legumelin, the method employed in preparing the material used for the hydrolysis here described, and also the literature of the proteins of the pea, are given in a recent paper from this laboratory,<sup>2</sup> they will not be repeated here.

The results of this hydrolysis show legumelin to be distinctly different in constitution from the other proteins with which it is associated in the pea. The properties and ultimate composition of legumelin closely resemble those of leucosin from the wheat embryo, and this resemblance extends not only to the general proportion of the several amino-acids, but also to the total quantity obtained from each of these albumins as may be seen from the accompanying table.

It is not improbable that legumelin is a constituent of the physiologically active tissues of the seed, rather than a reserve food substance for the developing seedling.

This supposition in regard to leucosin is supported by the fact that this albumin is located almost entirely in the embryo of the seed and that it resembles more closely, both in properties and composition, the proteins of physiologically active tissues of

<sup>1</sup> The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

<sup>2</sup> Osborne and Harris: *This Journal*, iii, p. 213, 1907.

animals, than those which unquestionably form the reserve food protein of the endosperm of other seeds.

As the leguminous seeds do not contain a sharply differentiated embryo and endosperm, but are composed of tissues which combine the functions of both, it is not possible to locate any one of their proteins in any particular part of these seeds. The just mentioned similarity, however, of legumelin to leucosin is suggestive of similarity also in their physiological functions.

The results of the hydrolysis of legumelin are given in the following table, and in order to facilitate the above comparison the results obtained for legumin, vicilin and leucosin are also given.

	Legumin pea per cent.	Vicilin pea per cent.	Legumelin pea per cent.	Leucosin wheat per cent.
Glycocoll.....	0.38	0.00	0.50	0.94
Alanine.....	2.08	0.50	0.92	4.45
Valine.....	?	0.15	0.69	0.18
Leucine.....	8.00	9.38	9.63	11.34
Proline.....	3.22	4.06	3.96	3.18
Phenylalanine.....	3.75	3.82	4.79	3.83
Aspartic acid.....	5.30	5.30	4.11	3.35
Glutaminic acid.....	16.97	21.34	12.96	6.73
Serine.....	0.53	?	?	?
Cystine.....	not det.	not det.	not det.	not det.
Oxyproline.....	"	"	"	"
Tyrosine.....	1.55	2.38	1.56	3.34
Arginine.....	11.71	8.91	5.45	5.94
Histidine.....	1.69	2.17	2.27	2.83
Lysine.....	4.98	5.40	3.03	2.75
Ammonia.....	2.05*	2.03*	1.26*	1.41*
Tryptophane.....	present	present	present	present
	<hr/> 62.22	<hr/> 65.44	<hr/> 51.13	<hr/> 50.53

\* Osborne and Harris: *Journ. Amer. Chem. Soc.*, xxv, p. 323, 1903.

### *Hydrolysis of Pea Legumelin.*

There were taken for this hydrolysis 550 grams, equal to 479.5 grams of ash and moisture-free legumelin. After warming for four hours with a mixture of 550 cc. of water and 550 cc. of hydrochloric acid (sp. gr. 1.19) on the boiling water-bath, the legumelin was finally dissolved. The hydrolysis was then com-



pleted by boiling for nineteen hours in an oil-bath. The solution was concentrated to a volume of about 700 cc. and saturated at  $0^{\circ}$  with dry hydrochloric acid gas. After standing on ice for one week, the separated glutaminic acid hydrochloride was filtered off. The yield, after deducting the ammonium chloride present, was equivalent to 50.45 grams glutaminic acid, or 10.52 per cent of the protein. This glutaminic acid hydrochloride, after dissolving in water and reprecipitating with strong hydrochloric acid, gave the following analysis:

*Chlorine*, 0.2127 gm. subst. gave 0.1695 gm. AgCl.

Calculated for  $C_5H_9O_4NHCl$  ..... Cl = 19.35 per cent.

Found..... Cl = 19.70 "

The filtrate from the glutaminic acid hydrochloride was concentrated, under reduced pressure, to a very heavy syrup which was esterified in the manner often described. After the esters had been shaken out with ether, the aqueous layer was freed from inorganic salts and the esterification was once more repeated. The combined ethereal extracts were thoroughly dehydrated with sodium sulphate and, after removing the ether at 760 mm. the residual amino-acid esters were fractioned under diminished pressure with the following results:

Fraction.	Temp. of bath up to	Pressure, mm.	Weight, grams.
I.....	$85^{\circ}$	23.	5.65
II.....	$80^{\circ}$	4.	37.81
III.....	$110^{\circ}$	0.85	97.27
IV.....	$136^{\circ}$	0.73	54.75
V.....	$195^{\circ}$	0.65	58.93
Total.....			254.41

The undistilled residue weighed 65 grams.

*Fraction I.* This fraction (including the ether removed at ordinary pressures) yielded 4.49 grams of glycocoll ethyl-ester hydrochloride, which at  $145^{\circ}$  melted sharply to a clear oil.

*Nitrogen*, 0.1974 gm. subst. required 14.3 cc.  $\frac{N}{10}$  HCl.

Calculated for  $C_4H_{10}O_2NCl$  ..... N = 10.04 per cent.

Found ..... N = 10.14 "

The free amino-acids in the filtrate from the glycocoll ethyl-ester hydrochloride were regenerated, and 0.50 gram of leucine and

0.90 gram of alanine were obtained. The alanine crystallized in needles having the following composition:

*Carbon and hydrogen*, 0.1739 gm. subst. gave 0.2583 gm. CO<sub>2</sub> and 0.1273 gm. H<sub>2</sub>O.

Calculated for C<sub>3</sub>H<sub>7</sub>O<sub>2</sub>N.....C = 40.45; H = 7.86 per cent.

Found.....C = 40.50; H = 8.13 "

*Fraction II.* This fraction was saponified by boiling with water for seven hours. The solution was then evaporated to dryness under diminished pressure, and the dried amino-acids were boiled with absolute alcohol, thus extracting the proline. After removing the proline, the remaining amino-acids, which weighed 23.75 grams, were subjected to a careful fractional crystallization. Glycocoll was not found. There were isolated, 9.95 grams of leucine, 3.31 grams of valine and 3.6 grams of alanine. The leucine weighed was analytically pure. The decomposition point was 298°.

*Carbon and hydrogen*, 0.1533 gm. subst. gave 0.3090 gm. CO<sub>2</sub> and 0.1356 gm. H<sub>2</sub>O.

Calculated for C<sub>6</sub>H<sub>10</sub>O<sub>2</sub>N.....C = 54.96; H = 9.92 per cent.

Found.....C = 54.98; H = 9.82 "

The valine when dissolved in 20 per cent hydrochloric acid had a specific rotation of  $(\alpha) \frac{20^\circ}{D} = +26.1^\circ$ .

*Carbon and hydrogen*, 0.1391 gm. subst. gave 0.2626 gm. CO<sub>2</sub> and 0.1162 gm. H<sub>2</sub>O.

Calculated for C<sub>5</sub>H<sub>11</sub>O<sub>2</sub>N.....C = 51.28; H = 9.40 per cent

Found.....C = 51.48; H = 9.28 "

The alanine crystallized in needles.

*Carbon and hydrogen*, 0.1471 gm. subst. gave 0.2191 gm. CO<sub>2</sub> and 0.1060 gm. H<sub>2</sub>O.

Calculated for C<sub>3</sub>H<sub>7</sub>O<sub>2</sub>N.....C = 40.45; H = 7.86 per cent.

Found.....C = 40.62; H = 8.00

Recrystallized once again from dilute alcohol, this alanine gave beautiful needles which decomposed at 290° on rapid heating.

*Carbon and hydrogen*, I, 0.2619 gm. subst. gave 0.3908 gm. CO<sub>2</sub> and 0.1889 gm. H<sub>2</sub>O. II, 0.1490 gm. subst. gave 0.2227 gm. CO<sub>2</sub> and 0.0996 gm. H<sub>2</sub>O.

Calculated for C<sub>3</sub>H<sub>7</sub>O<sub>2</sub>N: ..... C = 40.45; H = 7.86 per cent.

Found ..... I, C = 40.69; H = 8.01 "

II, C = 40.76; H = 7.42 "

*Fraction III.* This fraction was boiled with water for seven hours and the solution concentrated to dryness under reduced pressure. Proline was extracted from the dried residue with absolute alcohol. The amino-acids insoluble in alcohol weighed 45.71 grams, from which there were isolated 35.73 grams of leucine. The alcoholic extracts containing the proline from Fractions II and III were joined, and the alcohol removed under diminished pressure. The residue was extracted with boiling alcohol and the solution allowed to stand over night. After filtering out 1.15 grams of substance which had separated on standing, this process was repeated until nothing more was deposited after standing for several hours. The proline yielded 2.03 grams of the racemic copper salt, and 22.24 grams of the lævo-salt dried at 110°. The lævo-proline was coupled with phenylisocyanate, and the resulting phenylhydantoin melted sharply at 143° to a clear oil.

*Carbon and hydrogen*, 0.1162 gm. subst. gave 0.2340 gm. CO<sub>2</sub> and 0.0635 gm. H<sub>2</sub>O.

Calculated for C<sub>12</sub>H<sub>12</sub>O<sub>2</sub>N ..... C = 66.67; H = 5.57 per cent.

Found ..... C = 66.65; H = 6.07 "

The racemic salt, when once recrystallized from water, separated in the characteristic plates.

*Water*, I, 0.2429 gm. subst., air dried, lost 0.0268 gm. H<sub>2</sub>O at 110°

II, 0.6486 " " " " 0.0712 " " "

Calculated for C<sub>10</sub>H<sub>16</sub>O<sub>4</sub>N<sub>2</sub>Cu.2H<sub>2</sub>O ..... H<sub>2</sub>O = 10.99 per cent.

Found ..... H<sub>2</sub>O = I, 11.03 "

II, 10.99 "

*Copper*, 0.3538 gm. subst., dried at 110°, gave 0.0961 gm. CuO.

Calculated for C<sub>10</sub>H<sub>16</sub>O<sub>4</sub>N<sub>2</sub>Cu ..... Cu = 21.81 per cent.

Found ..... Cu = 21.70 "

*Fraction IV.* This fraction was suspended in five volumes of water and shaken with an equal volume of ether. The ether yielded, by the usual treatment, 9.90 grams of phenylalanine hydrochloride. The aqueous layer was saponified by warming on the water-bath for five hours with 100 grams of baryta. There were obtained 13.54 grams of aspartic acid as the barium salt. After once recrystallizing the free acid from water, the crystals reddened but did not decompose at 300°.

*Carbon and hydrogen,* 0.2400 gm. subst. gave 0.3211 gm. CO<sub>2</sub> and 0.1171 gm. H<sub>2</sub>O.

Calculated for C<sub>4</sub>H<sub>7</sub>O<sub>4</sub>N.....C = 36.09; H = 5.26 per cent.

Found.....C = 36.48; H = 5.42 “

The filtrate from the barium aspartate was quantitatively freed from barium and the solution was concentrated to a syrup under diminished pressure. No glutaminic acid could be separated from this solution as the hydrochloride. The free amino-acids were regenerated and converted into their copper salts, from which 2.93 grams of air dry copper aspartate were separated. The copper was removed from the filtrate from the copper aspartate, but no serine could be isolated from the solution.

*Fraction V.* This fraction yielded 18.07 grams of phenylalanine hydrochloride, 3.55 grams of aspartic acid, 11.69 grams of glutaminic acid hydrochloride and 2.54 grams of copper aspartate. The free phenylalanine was analyzed as follows:

*Carbon and hydrogen,* 0.1637 gm. subst., gave 0.3940 gm. CO<sub>2</sub>.

Calculated for C<sub>9</sub>H<sub>11</sub>NO<sub>2</sub>.....C = 65.45; H = 6.66 per cent.

Found.....C = 65.64; H = lost

The aspartic acid obtained from the barium salt reddened but did not decompose at 300°.

*Carbon and hydrogen,* 0.2553 gm. subst. gave 0.3377 gm. CO<sub>2</sub> and 0.1248 gm. H<sub>2</sub>O.

Calculated for C<sub>4</sub>H<sub>7</sub>O<sub>4</sub>N.....C = 36.09; H = 5.26 per cent.

Found.....C = 36.07; H = 5.43 “

The glutaminic acid hydrochloride after one recrystallization from strong hydrochloric acid, decomposed at 199°. With an equivalent quantity of potassium hydroxide, it yielded free glutaminic acid which decomposed sharply at 202° to 203°.

*Carbon and hydrogen*, 0.1252 gm. subst. gave 0.1866 gm.  $\text{CO}_2$  and 0.0700 gm.  $\text{H}_2\text{O}$ .

Calculated for  $\text{C}_8\text{H}_9\text{O}_4\text{N}$  ..... C = 40.81; H = 6.12 per cent.

Found ..... C = 40.64; H = 6.21 "

The copper aspartate crystallized from a large volume of water in the characteristic sheaves.

*Nitrogen*, 0.2558 gm. subst. required 9.7 cc.  $\frac{\text{N}}{1000}$  HCl.

Calculated for  $\text{C}_4\text{H}_5\text{O}_4\text{N Cu} \cdot 4\frac{1}{2}\text{H}_2\text{O}$  ... Cu = 23.07; N = 5.08 per cent.

Found ..... Cu = 23.26; N = 5.30 "

#### GLUTAMINIC ACID.

This protein resembles leucosin in regard to the difficulty of determining glutaminic acid directly, as is shown by the following.

Eighty-four grams of pea legumelin, equivalent to 73.23 grams ash and moisture-free, were hydrolyzed by boiling with a mixture of 84 cc. of water and 84 cc. of hydrochloric acid (sp. gr. 1.19) for twenty-six hours. The solution was sharply concentrated to a heavy syrup and saturated at  $0^\circ$  with hydrochloric acid gas. After prolonged standing on ice, 3.13 grams of glutaminic acid hydrochloride separated. The filtrate was again saturated and 2.15 grams further was obtained. This is 5.76 per cent of the protein, while we obtained in the hydrolysis of the larger quantity first described, 12.96 per cent.

#### THE RESIDUE AFTER DISTILLATION.

The undistilled residue which weighed 65 grams was dissolved in boiling absolute alcohol. On standing over night, 2 grams of substance separated, which was filtered out. After removing the alcohol and heating the residual solution with an excess of baryta for five hours, the baryta was removed, and glutaminic acid separated as the hydrochloride, in the usual manner. This weighed 2.91 grams and decomposed at  $199^\circ$ . The free acid decomposed at  $203^\circ$ .

#### TYROSINE.

A quantity of legumelin equal to 43.63 grams of ash and moisture-free substance was hydrolyzed with 150 grams of concentrated sulphuric acid and 300 cc. of water, by digesting on a boiling

water-bath until the legumelin was dissolved and then boiling the solution for 12 hours in an oil-bath. After removing the sulphuric acid and extracting the barium sulphate by boiling out with water, the solution was concentrated to crystallization. The substance that separated was then redissolved in boiling water, decolorized with animal charcoal and the tyrosine recrystallized by concentrating and cooling the solution. The tyrosine thus obtained weighed 0.68 gram, equal to 1.56 per cent.

*Carbon and hydrogen*, 0.1573 gm. subst. gave 0.3460 gm.  $\text{CO}_2$  and 0.0894 gm.  $\text{H}_2\text{O}$ .

Calculated for  $\text{C}_9\text{H}_{11}\text{O}_3\text{N}$  ..... C = 59.67; H = 6.08 per cent.

Found..... C = 59.98; H = 6.31 "

The filtrates from the tyrosine were used for determinations of the bases according to the method of Kossel and Patten.

#### CYSTINE.

No attempt was made to determine cystine, on account of lack of sufficient material.

#### HISTIDINE.

The solution of the histidine = 500 cc.

*Nitrogen*, 100 cc. solution required 5.36 cc.  $\frac{5\text{N}}{7}$  HCl = 0.2680 gm. N in 500 cc. = 0.9889 gm. histidine = 2.27 per cent.

The histidine was converted into the dichloride for identification.

*Chlorine*, 0.1000 gm. subst., air dry, gave 0.1252 gm. AgCl.

Calculated for  $\text{C}_6\text{H}_9\text{O}_2\text{HCl}$ ..... Cl = 31.14 per cent.

Found..... Cl = 30.96 "

The histidine dichloride decomposed at  $232^\circ$ .

#### ARGININE.

The solution of the arginine = 1000 cc.

*Nitrogen*, 50 cc. solution required 3.71 cc.  $\frac{5\text{N}}{7}$  HCl = 0.7420 gm. N in 1000 cc. = 2.3054 gm. arginine + 0.0720 gm. = 2.3774 gm. = 5.45 per cent.

The arginine was converted into the copper nitrate double salt for identification. The greater part of the solution was lost by an accident, therefore only a little substance was available.

*Copper*, 0.0366 gm. subst. air dry gave 0.0050 gm. CuO.

Calculated for  $C_{12}H_{28}O_4N_8 \text{ Cu (NO}_3)_2 \cdot 3H_2O$  . . Cu = 10.79 per cent.

Found.....

Calculated for  $C_{12}H_{28}O_4N_8 \text{ Cu (NO}_3)_2 \cdot 3H_2O$  . . Cu = 10.79 per cent.

Found..... Cu = 10.92 "

#### LYSINE.

The lysine picrate weighed 3.400 grams = 1.3224 grams lysine = 3.03 per cent. The lysine was identified as the picrate.

*Nitrogen*, 0.3000 gm. subst., dried at 100°, required 5.58 cc.  $\frac{5N}{7}$  HCl.

Calculated for  $C_6H_{14}O_2N_2 \cdot C_6H_3O_7N$ ..... N = 18.70 per cent.

Found..... N = 18.60





# THE DETECTION AND QUANTITATIVE DETERMINATION OF $\beta$ -OXYBUTYRIC ACID IN THE URINE.<sup>1</sup>

By O. F. BLACK

(Contribution from the Laboratory of Biological Chemistry of the Harvard Medical School.)

(Received for publication, July 29, 1908).

It is generally recognized that  $\beta$ -oxybutyric acid, on oxidation in the body, yields first acetacetic acid, which then decomposes into acetone. This reaction can be carried out in the laboratory by the use of hydrogen peroxide as the oxidizing agent, and was observed by me some months before a description of it was recently published by Dakin.<sup>2</sup> The intermediate product, acetacetic acid, can be tested for in a solution by the addition of ferric chloride which produces a bright red color, the well-known Gerhardt's test. If  $\beta$ -oxybutyric acid be tested with hydrogen peroxide in the presence of ferric chloride one obtains this same color reaction, which thus becomes a test for  $\beta$ -oxybutyric acid.

*Procedure.* Two or three drops of the ordinary commercial hydrogen peroxide are added to the dilute solution of  $\beta$ -oxybutyric acid in a test tube and mixed by shaking, then a few drops of 5 per cent ferric chloride containing a trace of ferrous chloride. On standing a few seconds a beautiful rose color develops which slowly intensifies until it reaches a maximum and then gradually fades owing to the further oxidation of the acetacetic acid. The chief precautions to be observed in carrying out the test are to be sure that the solution is cold and nearly neutral, and to avoid a large excess of hydrogen peroxide and iron. If too much of the oxidizing agents is added and but little  $\beta$ -oxybutyric acid be present the color developed is transitory or fails to appear. By starting with a small quantity and then adding more ferric chloride at intervals of a few minutes until no further color is produced one is able to observe the full intensity of color, and thereby get a rough idea as to the amount of  $\beta$ -oxybutyric acid

<sup>1</sup> Done with aid from the Proctor Fund.

<sup>2</sup> This *Journal*, iv, p. 1.

present. The test has a considerable delicacy; a solution containing 0.1 of a milligram per cubic centimeter, or one part in 10,000, gave an easily recognized color.

*In the urine.* The color of urine and the presence of acetacetic acid and sugar all interfere with the test when applied to the urine directly. The following procedure, however, has been found to give the required result, and can easily and quickly be carried out. Five to 10 cc. of the urine under examination are concentrated in an evaporating dish at a gentle heat to one-third or one-fourth of the original volume which eliminates the acetacetic acid. The residue is then acidified with a few drops of concentrated hydrochloric acid and made to a thick paste with plaster of paris and allowed to stand until it begins to set. It is then stirred and broken up in the dish with a blunt ended stirring rod. The porous meal thus obtained is extracted twice with ether by stirring and decantation. The ether extract which contains  $\beta$ -oxybutyric acid is evaporated spontaneously or on the water-bath. The residue is finally dissolved in water, neutralized with barium carbonate and the test applied as already described.

The reaction occurring in this test, namely, the oxidation of  $\beta$ -oxybutyric acid to acetacetic acid and thence to acetone has been investigated in this laboratory in its quantitative aspect with the hope that it might offer a means for the estimation of  $\beta$ -oxybutyric acid. The results were not encouraging for, although a certain degree of constancy was observed in the amounts of acetone produced by known quantities of  $\beta$ -oxybutyric acid, they fell far short of what was required by calculation. Moreover, sugar was found to yield products which interfered. So it was concluded that oxidation by hydrogen peroxide is not suited for a quantitative method.

#### QUANTITATIVE.

As described above in the qualitative method  $\beta$ -oxybutyric acid is readily extracted from urine after evaporating and drying with plaster of paris. It seemed probable that this might offer a means for the quantitative estimation of the acid more rapid than the methods that are now in use. Of the numerous methods proposed for determining this acid the most reliable are undoubtedly Geelmuyden's and Magnus-Levy's. These authors extract the urine, after strongly acidifying with a mineral acid, with ether in a continuous extractor for periods ranging from 48 to 72

hours. I have found that by previously drying the urine with plaster of paris a more complete extraction may be accomplished in two hours.

A known solution of active  $\beta$ -oxybutyric acid was prepared by concentrating some diabetic urine, acidifying the residue, drying with plaster of paris, and extracting with ether. This is an effective method for the preparation of the acid as fairly large volumes of urine can be concentrated and the product is but slightly discolored. The yellow syrup obtained was taken up with water decolorized with bone black, and its strength determined by the polariscope. This was used as a known solution to add to a normal urine as follows: 50 cc. of a normal urine was treated with a definite amount of the above solution of  $\beta$ -oxybutyric acid, made faintly alkaline with sodium carbonate and boiled down to one-third of its volume and then concentrated to about 10 cc. on the water-bath. The residue was cooled, acidified with a few drops of concentrated hydrochloric acid until it reddened litmus distinctly, and mixed with plaster of paris to a thick paste. The mixture on standing a few minutes begins to harden. It is then stirred and broken up with a stout glass rod and more plaster is added, if necessary, until it has the consistency of a fairly dry, coarse meal. This is transferred to a continuous extraction apparatus where it is extracted with ether for two hours. The ether extract is then evaporated, either spontaneously, or in an air current, the residue taken up with water, treated with a little bone black if necessary, filtered until perfectly clear and made to known volume (25 cc. or less). The  $\beta$ -oxybutyric acid is determined with the polariscope. The following figures were obtained with normal urines treated with known quantities of the acid.

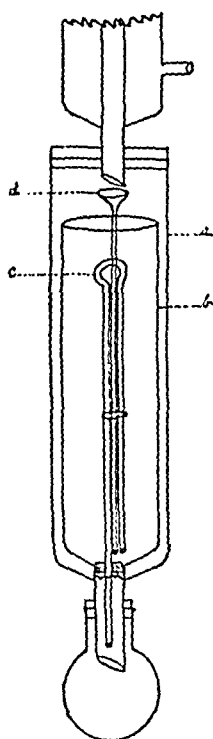
	Polariscope.	Calculated grams of acid.	Grams acid added.
1.....	1.49°	0.8150	0.8053
2.....	1.43°	0.7824	0.8053
3.....	1.46°	0.7990	0.8053
1.....	0.70°	0.3830	0.3840
2.....	0.70°	0.3830	0.3840
3.....	0.68°	0.3720	0.3840
1.....	1.02°	0.5590	0.5760
2.....	1.00°	0.5470	0.5760

A series of determinations on a diabetic urine containing much sugar gave these figures in grams.

	1.	2.	24-hour urine.
January 26.....	27.3	29.7	3720
January 27.....	28.0	29.3	3940
January 28.....	24.6	23.9	3210
January 29.....	26.7	25.5	3210
January 30.....	34.6	32.0	3190
January 31.....	50.5	52.0	4600
February 1.....	38.8	39.5	4050
February 2.....	36.5	37.0	3510

For purposes of comparison the method of Magnus-Levy was tried on one of the above urines, that of January 31 and two determinations which were extracted, one for 60 hours, and the other for 80 hours, gave respectively 48.8 and 45.5 grams, as against the 50.5 and 52.0 grams obtained by the plaster of paris method in a two-hour extraction.

A Soxhlet apparatus was used in the extraction, and also a modification of it which I have devised for this particular process



and which proved very effective. This apparatus consists of an outer jacket (*a*) fitted to a small flask and to a condenser. Inside the jacket is a tube of slightly smaller bore (*b*) with a one-hole cork stopper in the lower end, through which passes the siphon (*c*) to which is attached the funnel (*d*) by a twist of platinum wire. A pad of glass wool is packed about the open ends of the siphon and funnel in the lower part of (*b*) then the material to be extracted is poured upon it, not filling over the top of the siphon. The advantages of this apparatus are, first, that the inner tube is constantly exposed to the warm vapors of ether, then, that the ether returns to the boiling flask hot so there is no interruption in boiling as in a Soxhlet, finally that by using siphons of various lengths large or small amounts of substance may be extracted with little ether.

# A METHOD FOR THE QUANTITATIVE DETERMINATION OF $\beta$ -OXYBUTYRIC ACID IN URINE.

By PHILIP A. SHAFFER.

(From the Department of Experimental Pathology, Cornell University Medical College, New York.)

(Received for publication, August 8, 1908.)

We have at present for the determination of  $\beta$ -oxybutyric acid, no method which is not open to serious criticism, either on the point of accuracy or on account of time, labor and chemicals involved. The method of separately determining the inorganic acids and bases of the urine, and so calculating the amount of organic acid present, was first used for this purpose by Stadelmann<sup>1</sup> who adopted it from Gähtgens,<sup>2</sup> and from its use Stadelmann was led to the discovery of the organic acid, thus verifying the statement made three years earlier by Hallervorden that the high ammonia content of diabetic urines is due to the presence of an organic acid.<sup>3</sup>

This procedure has since been used in the study of  $\beta$ -oxybutyric acid in relation to acidosis by Magnus-Levy<sup>4</sup> and others. The method is very laborious, necessarily inaccurate, on account of the many operations required, and still more important, it does not determine the amount of  $\beta$ -oxybutyric acid, but merely the amount of total organic acidity. And for this latter purpose the simpler and more accurate method proposed by Folin<sup>5</sup> is to

<sup>1</sup> Stadelmann: *Arch. f. exp. Path. u. Pharm.*, xvii, p. 419, 1883.

<sup>2</sup> Gähtgens: *Zeitschr. f. physiol. Chem.*, iv, p. 36, 1880.

<sup>3</sup> Hallervorden (*Arch. f. exp. Path. u. Pharm.*, xii, p. 237) in 1880 confirmed the finding of Boussingault of high ammonia excretion in diabetes, and suggested that it was caused by the excretion of an organic acid (lactic or glycuronic acids). Stadelmann in 1883 confirmed Hallervorden and isolated crotonic acid. In 1884 Külz, *Zeitschr. f. Biol.*, xx, p. 165; *Arch. f. exp. Path. u. Pharm.*, xviii, p. 290, and Minkowski, *Arch. f. exp. Path. u. Pharm.*, xviii, pp. 35 and 147, independently showed the acid to be  $\beta$ -oxybutyric.

<sup>4</sup> Magnus-Levy: *Arch. f. exp. Path. u. Pharm.*, xlii, p. 149, 1899.

<sup>5</sup> Folin: *Amer. Journ. of Physiol.*, ix, p. 265, 1903.

be preferred. With one exception, the other methods for the determination of  $\beta$ -oxybutyric acid are based upon the optical activity of the lævorotary acid or its salts.

The rotation of the fermented urine is read in the polariscope (Külz) with or without preliminary treatment with basic lead acetate; or the acid is extracted by ether from the evaporated urine, and the rotation of an aqueous solution of the residue from the ether is determined, and the amount of oxybutyric acid calculated.

Direct reading of the fermented urine is easy enough but the results are worthless because of, first, the great percentage error in reading dilute solutions of the acid or its salts;<sup>1</sup> second, the probable presence of other optically active substances in urine even after fermentation; and third, if basic lead acetate be used, the action of this substance in increasing the lævorotation of salts of the acid (Magnus-Levy).<sup>2</sup> Extraction of the acid by ether, and subsequent polarization of the aqueous solution of the residue from the ether was apparently first used by Wolpe<sup>3</sup> in 1886. Without altering the principle of the method, it has been very materially improved by Magnus-Levy, Bergell,<sup>4</sup> and most recently by Black. With the use of the latter's modifications which consist in dehydrating the evaporated urine with plaster of paris, and the use of an improved continuous ether-extraction apparatus,<sup>5</sup> the method is fairly quick and the results may be fairly satisfactory. The principle of the method is still however open to the objection of difficulty of complete extraction, and that other optically active substances may be extracted from the urine,

<sup>1</sup> The specific rotation of the free acid is  $-24.12^\circ$  and of the sodium salt  $-14.35^\circ$  (Magnus-Levy: *Arch. f. exp. Path. u. Pharm.*, xlv, p. 393 and 397, 1901). The specific rotation is different for the salts with different bases. Magnus-Levy points out that an error of  $0.10^\circ$  in reading the polariscope would amount to about 15 grams in 5 liters of urine (*ibid.*, xlii, p. 170, 1899). An error of  $0.10^\circ$  in reading a 2 per cent solution of  $\beta$ -oxybutyric acid (which is frequently obtained from urines containing only a little of this acid) would amount to more than 10 per cent of the total.

<sup>2</sup> Magnus-Levy: *Arch. f. exp. Path. u. Pharm.*, xlv, p. 393, 1901.

<sup>3</sup> Wolpe: *Arch. f. exp. Path. u. Pharm.*, xxi, p. 138.

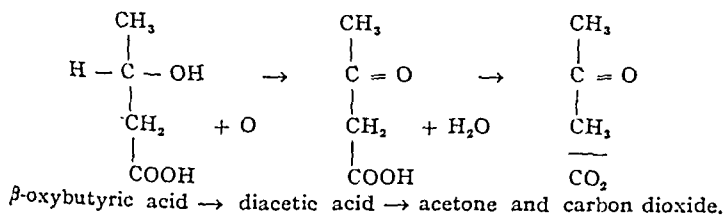
<sup>4</sup> Bergell: *Zeitschr. f. physiol. Chem.*, xxxiii, p. 310, 1901.

<sup>5</sup> Bergell used anhydrous copper sulphate and extracted in a Soxhlet apparatus.

and either increase or decrease the lævorotation of the final solution.

The method proposed by Darmstädter<sup>1</sup> is, from its author's description, very easy and exceedingly accurate; though in my hands it has not been successful. After evaporating the urine made alkaline with sodium carbonate Darmstädter distills it with a constant concentration of 50 per cent sulphuric acid thus converting the  $\beta$ -oxybutyric acid into crotonic acid which distills over. The distillate of 300 to 400 cc. is extracted *three* times with ether which, he claims, removes all of the crotonic acid. The ether is distilled and the residue, containing the crotonic acid, after being heated on a sand-bath to remove the volatile acids, is dissolved in water and titrated with alkali. Darmstädter in this way obtained results from 99.36 per cent to 99.70 per cent of the amount of synthetic  $\beta$ -oxybutyric acid added to urine. Unfortunately he does not state just how he determined so accurately the amount of oxybutyric acid used in his experiments. Of the various objections to this method as its author describes it, the most obvious is perhaps the difficulty in completely extracting the crotonic acid from 300 cc. of liquid, by two or three portions of ether. The method has not apparently been received with favor.

It occurred to me that it might be possible to utilize as the basis for a new method a property of  $\beta$ -oxybutyric acid long ago mentioned by Minkowski—its oxidation with the formation of acetone and carbon dioxide. I hoped that the reaction might, under certain conditions, proceed after the following well known scheme.



Distilling with sulphuric acid and potassium bichromate, under the conditions to be described, it is easily possible to obtain from  $\beta$ -oxybutyric acid the theoretical amount of acetone, the quantity

<sup>1</sup> Darmstädter: *Zeitschr. f. physiol. Chem.*, xxxvii, p. 355. 1903.

of which may be accurately determined by means of standard iodine and thiosulphate solutions.

The conditions necessary for a maximum yield of acetone concern the concentrations of sulphuric acid, and of bichromate. Too little sulphuric acid, even with an excess of bichromate liberates the acetone very slowly and perhaps incompletely; while a very great excess of sulphuric acid decomposes  $\beta$ -oxybutyric acid with the formation of crotonic acid. The optimum concentration appears to be between 3 per cent and 7 per cent sulphuric acid. The following experiments show the effect of varying amounts of sulphuric acid upon the speed with which the acetone is formed.

The determinations were made in a fairly pure solution of inactive  $\beta$ -oxybutyric acid, made by the reduction of acetacetic ester by sodium amalgam (Wislicenus).<sup>1</sup> About 90 per cent of the titrated acidity of the solution was due to oxybutyric acid (from the determination of acetone under conditions giving the correct results).

The volume of each distillate was about 300 cc.; more water was added to the distilling flask before each subsequent distillation or the volume was kept constant at about 500 cc. by means of water from a dropping funnel.

The acetone in the distillates was determined as usual with standard (103.5 per cent  $\frac{N}{10}$ ) thiousulphate and iodine solutions, of which 1 cc. = 1 mg. acetone. The results are given in milligrams of acetone.

With 1.0 gram potassium bichromate:

I.	1 cc. $H_2SO_4$ .....	1st dist.	16.0 mg. acetone.	
		2d "	18.8	"
		3d "	7.0	"
		4th "	2.4	"
			<hr/> 44.8	"
II.	2 cc. $H_2SO_4$ .....	1st dist.	21.5	"
		2d "	19.0	"
		3d "	6.2	"
		4th "	1.7	"
			<hr/> 48.4	"
III.	5 cc. $H_2SO_4$ .....	1st dist.	39.1	"
		2d "	10.6	"
			<hr/> 49.7	"

<sup>1</sup> Wislicenus: *Ann. d. Chem.*, cxlix, p. 205, 1869.



IV.	20 cc. $H_2SO_4$ .....	1st dist.	47.0	mg. acetone.
		2d	"	0.0
			<hr/>	
			47.0	"
V.	50 cc. $H_2SO_4$ .....	1st dist.	40.6	"
		2d	"	0.0
			<hr/>	
			40.6	"
VI.	50 cc. $H_2SO_4$ .....	1st dist.	43.0	"
		2d	"	0.0
			<hr/>	
			43.0	"

For bichromate the danger lies in use of too large a quantity, which gives very low results: probably because of a further oxidation of the acetacetic acid, first formed. This danger may be averted by adding the potassium bichromate in a dilute solution from a dropping funnel during the distillation. At the same time the concentration of sulphuric acid is thereby kept practically constant.

The following results show the effect of varying quantities of potassium bichromate; in these experiments both sulphuric acid and bichromate were added before starting the distillation. The original volume in each distilling flask was about 500 cc. of which about 300 to 350 cc. was distilled.

With 10 cc.  $H_2SO_4$

I.	With 0.3 gm. $K_2Cr_2O_7$	=	40.0	mg. acetone.
II.	0.5 "	"	40.0	"
III.	0.5 "	"	41.0	"
IV.	1.0 "	"	36.0	"
V.	1.0 "	"	35.3	"
VI.	3.0 "	"	36.8	"
VII.	5.0 "	"	23.7	"

With 15 cc.  $H_2SO_4$

I.	With 1 gm. $K_2Cr_2O_7$	36.2	"
II.	5 "	23.5	"
III.	10. "	20.5	"
IV.	20 " (20 cc. $H_2SO_4$ )	13.9	"

The following are the results of similar determinations except that the bichromate (in dilute solution, usually 0.2 to 0.5 per cent) was added from a dropping funnel during the distillation; the solution dropping in about as fast as the distillate collected.

I.	10 cc. $H_2SO_4$	0.5	gm.	$K_2Cr_2O_7$	40.0 mg. acetone.
II.	10	"	0.5	"	"
III.	7	"	0.5	"	"
IV.	7	"	0.5	"	"
V.	10	"	3.0	"	"
VI.	10	"	3.0	"	"
VII.	10	"	3.0	"	"
VIII.	10	"	3.0	"	"
IX.	10	"	3.0	"	"
X.	20	"	3.0	"	"

In a different solution:

									Mg. acetone per 100 cc. oxy- butyric acid solution.
I.	20 cc. oxybutyric acid sol.				15 cc. $H_2SO_4$	1 %	$K_2Cr_2O_7$		84.0
II.	40	"	"	"	15	"	1	"	84.5
III.	20	"	"	"	15	"	1	"	81.5
IV.	40	"	"	"	15	"	1	"	79.5
V.	20	"	"	"	20	"	2	"	78.5
VI.	40	"	"	"	20	"	2	"	79.3
VII.	20	"	"	"	20	"	2	"	82.5
VIII.	40	"	"	"	20	"	2	"	83.0
IX.	20	"	"	"	20	"	0.1	"	82.5
X.	20	"	"	"	20	"	2.	"	79.5

These results show that even when a dropping funnel is used for the addition of the bichromate, the solution should not be too concentrated, or, what is the same thing, the bichromate should be added *slowly*. When this is done the results are accurate. In order to prove this point sodium salt of the inactive acid (already about 90 per cent pure) was prepared and recrystallized three times from absolute alcohol. This recrystallized salt was dissolved in water, excess of sulphuric acid added, cooling with ice, and the solution dehydrated with plaster of paris. The plaster was powdered and extracted with dry ether in a Soxhlet apparatus. The aqueous solution of the ether residue was boiled with pure bone black, filtered, cooled and a portion titrated with  $\frac{N}{10}$  alkali (phenolphthalein).

25 cc. = 6.85 cc.  $\frac{N}{10} \times 10.4 = 71.2$  mg.  $\beta$ -oxybutyric acid = 39.6 mg. acetone.

Five determinations under identical conditions in 25 cc. portions of this solution gave

40.2 mg. acetone	=	101.3 per cent.
39.1 " "		98.8 "
41.0 " "		103.3 "
40.7 " "		102.7 "
39.2 " "		99.0 "

The distillations were carried out with 15 cc. of concentrated sulphuric acid and keeping the volume nearly constant at about 400 cc. by dropping in water or 0.2 per cent potassium bichromate from a dropping funnel. A total of about 1 gram of potassium bichromate was added, and about 800 cc. distilled in each case.

When this method was applied to urine, difficulties were at once encountered, but were satisfactorily overcome.

Normal urines distilled with chromic acid give acetone although in small quantities. This was shown in 1885 by Flückiger,<sup>1</sup> who found the chief source of the acetone in the conjugated glucuronic acids. The glucuronic acids may however be removed by basic lead acetate and ammonia, which reagents do not precipitate  $\beta$ -oxybutyric acid. The small quantities of formic and butyric acids present in normal and pathological urines would interfere with the determination of  $\beta$ -oxybutyric acid by this method, but these volatile acids may be removed by distilling the urine with sulphuric acid before the addition of bichromate.<sup>2</sup>

<sup>1</sup> Flückiger: *Zeitschr. f. physiol. Chem.*, ix, p. 343.

<sup>2</sup>  $\beta$ -Oxybutyric acid in the concentration present during the distillation (never over 0.05 per cent) is not decomposed by 1 per cent to 10 per cent sulphuric acid. Ten cc.  $\beta$ -oxybutyric acid solution + 500 cc. water + 15 cc. sulphuric acid was distilled until 200 cc. distillate had collected. This distillate contained 0.35 cc.  $\frac{N}{10}$  acidity, the equivalent of 3.6 mg. of  $\beta$ -oxybutyric acid. The liquid in the distilling flask was again distilled, this time dropping in 0.5 per cent potassium bichromate. This distillate contained 108.4 mg. acetone. Duplicate determinations in the same oxybutyric acid solution, but without previous boiling with sulphuric acid, gave 107.8 mg. and 108.6 mg. acetone. Araki: *Zeitschr. f. physiol. Chem.*, xviii, p 1, 1893) found on distilling 200 cc. of a 1 per cent solution of  $\beta$ -oxybutyric acid that about 1 per cent (0.019 gm.) passed over as crotonic acid in the first 100 cc. distillate.

Any formic acid passing into the distillate containing the acetone from  $\beta$ -oxybutyric acid would use up iodine in the subsequent titration; while butyric acid, on treatment with bichromate is partially oxidized, presumably to  $\beta$ -oxybutyric acid, thus increasing the yield of acetone.

Glucose also presents obstacles, since on treatment with chromic acid small quantities of what I believe to be formic aldehyde and formic acid are formed. This trouble is very easily overcome by redistilling the distillate supposed to contain acetone from  $\beta$ -oxybutyric acid, with hydrogen peroxide and alkali. The acetone is not attacked<sup>1</sup> but the aldehyde (?) is oxidized to the corresponding acid, which is held back by the alkali.

Lactic acid, when present, also interferes, presumably by the formation of acetic aldehyde which passes over with the acetone, and yields iodoform on treatment with hypoiodite; but this difficulty is likewise wholly removed by subsequent distillation with hydrogen peroxide and alkali.

The following experiments illustrate the above statement concerning glucose and lactic acid.

2 grams glucose in 500 cc. water + 10 gm. sulphuric acid distilled, dropping in 3 per cent potassium bichromate (12 gm.). 600 cc. of the distillate titrated with iodine and thiosulphate gave the equivalent of 19.7 mg. acetone; *but no iodoform was found, the solution remaining clear.*

1 gram glucose in 500 cc. water + 5 cc.  $\beta$ -oxybutyric acid solution + 10 cc. sulphuric acid. Distilled, dropping in 3 per cent potassium bichromate (9 gm.). Distillate = 46.2 mg. acetone. Duplicate = 47.3 mg. acetone.

No glucose, 5 cc.  $\beta$ -oxybutyric acid solution, etc. Distillate = 36.7 mg. acetone. Duplicate = 38.7 mg. acetone.

1 gm. glucose + 50 cc. of a different  $\beta$ -oxybutyric solution + 10 cc. sulphuric acid + water. Distilled, dropping in 2 per cent potassium bichromate. First distillate redistilled with 25 cc. 3 per cent hydrogen peroxide + 5 cc. 10 per cent sodium hydroxide. This distillate = 41.6

---

<sup>1</sup> Dakin: This *Journal*, iv, p. 81, 1908. The following experiment shows the stability of acetone in the presence of hydrogen peroxide. 25 cc. of a dilute acetone solution titrated direct with standard iodine and thiosulphate = 36.3 mg. acetone. 25 cc. acetone solution + 400 cc. water + 25 cc. 3 per cent hydrogen peroxide + 5 cc. 10 per cent sodium hydroxide. Distillate contained 36.3 mg. acetone. Duplicate distillate contained 36.9 mg. acetone.

mg. acetone. Control determination in pure solution without glucose = 42.8 mg. acetone.

2.5 gms. calcium lactate in 500 cc. water + 10 cc. sulphuric acid. Distilled, dropping in 2 per cent potassium bichromate. 400 cc. of the distillate gave the equivalent of 80 mg. acetone, with the formation of much iodoform.

1 gm. calcium lactate in 500 cc. water + 25 cc. acetone solution (= 36.3 mg. acetone) + 10 cc. sulphuric acid. Distilled 500 cc., dropping in 2 per cent potassium bichromate (4 gms.)

(a) 125 cc. distillate titrated direct =  $18.3 \times 4 = 73.2$  mg. acetone.

(b) 250 cc. distillate redistilled with hydrogen peroxide + sodium hydroxide =  $17.9 \times 2 = 35.8$ .

(c) Gave strong positive reaction with ammoniacal silver nitrate solution containing sodium hydroxide.

By the use of basic lead acetate and ammonia, and of the second distillation with hydrogen peroxide and alkali it is possible to overcome all of the more serious drawbacks to this method which I have so far encountered.

Phenol and skatol from the conjugated acids may still cause some inaccuracy but the greater part of these substances pass over in the preliminary distillation with sulphuric acid alone; and in any event this error is very small—from 20 to perhaps 100 mg. in a 24-hour urine.

Other substances, such as leucin,<sup>1</sup> sometimes present in urine in relatively small quantities, may be capable of forming acetone under the circumstances, but it is not likely that this possible error is of sufficient size to materially detract from the usefulness of the method.

A number of different normal and pathological urines, not containing  $\beta$ -oxybutyric acid, which I have examined have given results equivalent to less than 0.100 gram  $\beta$ -oxybutyric acid for a 24-hour urine (0.010 gram to 0.080 gram).

In the routine use of this method we determine at the same time the preformed acetone plus acetone from diacetic acid, with very satisfactory results. The three distillations of the Messenger-Huppert method are for practical purposes unnecessary, though two distillations are desirable.

The method which I propose for the determination of acetone + diacetic acid, and of  $\beta$ -oxybutyric acid is carried out as follows:

<sup>1</sup> Dakin: This *Journal*, iv, p. 63, 1908.

From 25 to 250 cc. of urine, depending upon whether much or little  $\beta$ -oxybutyric acid is expected,<sup>1</sup> is measured into a 500 cc. volumetric flask and an excess of basic lead acetate and 10 cc. of concentrated ammonium hydroxide are added. This is diluted to the mark with water, shaken and filtered. An aliquot part of the filtrate (usually 200 cc.) is diluted with water to 500 to 600 cc., 15 cc. of concentrated sulphuric acid and talcum added, and the mixture distilled until 200 to 250 cc. of distillate has collected. (Distillate A.)

The distilling flask (800 cc. Kjeldahl's are convenient) must be fitted with a dropping tube and water run in to prevent the volume in the flask from becoming less than 400 cc.

Distillate A contains the acetone, preformed and from diacetic acid, and also the volatile fatty acids present; to remove the latter, including the disturbing formic acid, distillate A is redistilled after adding a little fixed alkali (5 cc. of 10 per cent sodium hydroxide). This distillate ( $A_2$ ) is titrated with standard iodine and thiosulphate.

The residue of urine + sulphuric acid from which Distillate A was obtained is again distilled, dropping in 400 to 600 cc. of 0.1 per cent to 0.5 per cent potassium bichromate solution. (Ordinarily 0.5 gm. potassium bichromate will be sufficient; with much sugar or when much urine is used even 2 or 3 gms. may be necessary.) The potassium bichromate solution should not be added faster than the distillate collects unless the boiling liquid turns pure green, thus indicating that the bichromate is being used up more rapidly. When about 500 cc. of distillate (B) has collected, 20 cc. of 3 per cent hydrogen peroxide is added to the distillate (or the distillate may be collected in a flask containing the hydrogen peroxide) together with a few cc. of sodium hydroxide solution and this distillate (B) is again distilled. This dis-

<sup>1</sup> Of diabetic or other urines giving a strong ferric chloride reaction for diacetic acid, or when 5 to 10 grams or more  $\beta$ -oxybutyric acid is expected, 25 to 50 cc. or even less urine will be found a suitable quantity; when little or no  $\beta$ -oxybutyric acid is expected, 125 cc. or 250 cc. may be used. In either case the amount taken is sufficient for duplicate determinations. The aim should be to use such a quantity of urine as will give between 25 and 50 mg. of acetone from  $\beta$ -oxybutyric acid.

tillate now obtained ( $B_2$ ; 300 cc. should be distilled) is titrated as usual with iodine and thiosulphate.

A good condenser must be used for the distillations, but it is not necessary to cool the distillates with ice, as is sometimes recommended.

We find it convenient to make our thiosulphate and iodine solution 103.4 per cent  $\frac{N}{10}$ . Each cc. of the iodine solution is then equal to 1 mg. of acetone or to 1.794 mg. of  $\beta$ -oxybutyric acid. The thiosulphate is accepted as the standard, and is restandardized from time to time by  $\frac{N}{10}$  potassium bi-iodate.

A few determinations in urines containing no  $\beta$ -oxybutyric acid and also of normal urines to which were added varying amounts of synthetic  $\beta$ -oxybutyric acid, are given below.

Lab. No. 622 Mixed urine of 5 days from a case of epilepsy. Average for 24 hours.

Acetone + diacetic	=	0.018 gm. acetone.
$\beta$ -Oxybutyric acid	=	0.018 "
		0.025 "

Lab. No. 626. 24-hour urine from a case of Grave's disease. 1220 cc. sp. gr. 1.017.

Totals: Acetone + diacetic	=	0.015 gm. acetone.
$\beta$ -Oxybutyric	=	0.025 "

Lab. No. 637. 24-hour urine from woman 5 months pregnant.

Totals: Acetone + diacetic	=	0.032 gm. acetone.
$\beta$ -Oxybutyric	=	0.032 "

Lab. No. 638. 24-hour urine from woman 7 months pregnant, who had a moderate acidosis some weeks earlier.

Acetone + diacetic	=	0.006 gm. acetone.
$\beta$ -Oxybutyric	=	0.007 "

Lab. No. 641. Specimen of urine representing probably about 12 hours from a case of puerperal eclampsia. Urine passed about the time of the convulsions 400 cc., sp. gr. 1.019; 0.25 per cent albumen.

Acetone + diacetic	=	0.027 gm. acetone.
$\beta$ -Oxybutyric	=	0.042 "

Mixed normal urine.

For 1000 cc.:  $\beta$ -oxybutyric = 0.025 gm. acetone.

To the same urine was added 1.50 gm.  $\beta$ -oxybutyric acid per liter of urine.

Determination of  $\beta$ -Oxybutyric Acid

For 1000 cc. of urine, $\beta$ -oxybutyric acid	=	0.860 gm. acetone.
" " " " "		0.865 "
" " " " "		0.890 "
" " " " "		0.860 "

Average = 0.869 gm. acetone.

= 1.56 gm.  $\beta$ -oxybutyric acid.

Determinations in same  $\beta$ -oxybutyric acid solution: (a) 0.825 gm.;  
(b) 0.830 gm.; (c) 0.840 gm.; (d) 0.840.

Average = 0.834 gm. acetone,

= 1.50 gm.  $\beta$ -oxybutyric acid.

Normal urine; sp. gr. 1.021.

Results are given in grams of acetone per 1000 cc. of urine.

I. Without treatment with basic lead acetate and ammonia.

Acetone + diacetic: Distillate A titrated direct = 0.060 gm.

Distillate A redistilled with

sodium hydroxide = 0.016 "

$\beta$ -Oxybutyric: Distillate B titrated direct = 0.28 "

Distillate B redistilled with

hydrogen peroxide and  
sodium hydroxide = 0.18 "

II. After treatment with basic lead acetate and ammonia.

Acetone + diacetic: Distillate A titrated direct = 0.042 "

Distillate A redistilled with

sodium hydroxide = 0.008 "

$\beta$ -Oxybutyric: Distillate B titrated direct = 0.046 "

Distillate B redistilled with

hydrogen peroxide and  
sodium hydroxide = 0.018 "

III. Same urine +  $\beta$ -oxybutyric acid solution (2000 cc.  $\beta$ -oxybutyric acid solution contained according to determinations in the solution 0.686 gm. acetone). 2000 cc. of  $\beta$ -oxybutyric solution to 1000 cc. urine. Treated with basic lead acetate and ammonia.

Acetone + diacetic: Distillate A titrated direct = 0.036 "  
0.036 "

Distillate A redistilled with

sodium hydroxide. = 0.010 "

$\beta$ -Oxybutyric: Distillate B titrated direct = 0.706 "

0.668 "

Distillate B redistilled with

hydrogen peroxide and  
sodium hydroxide = 0.670 "

0.634 "



- IV. Same proportions of urine and  $\beta$ -oxybutyric acid as in III; 8 gms. glucose added per 100 cc. urine. Treated with basic lead acetate and ammonia.

Acetone + diacetic: Distillate A redistilled with sodium hydroxide = 0.016 gm.

$\beta$ -Oxybutyric acid: Distillate B redistilled with  $H_2O_2 + N_2OH$  = 0.664 "

- V. Same as III, but 4 gms. calcium lactate added per 100 cc. urine. Treated with basic lead acetate and ammonia.

Acetone + diacetic: Distillate A titrated direct = 0.042 "

Distillate A redistilled with sodium hydroxide = 0.022 "

$\beta$ -Oxybutyric: Distillate B titrated direct = 2.338 "

Distillate B redistilled with hydrogen peroxide and sodium hydroxide = 0.664 "

- VI. Same urine + one-half the amount of  $\beta$ -oxybutyric acid (= 0.343 gm. acetone per 1000 cc. urine, according to previous determinations in pure solution). Treated with basic lead acetate and ammonia.

Acetone + diacetic: Distillate A redistilled with sodium hydroxide. = 0.014 "

$\beta$ -Oxybutyric: Distillate B redistilled with hydrogen peroxide and sodium hydroxide. = 0.330 "

0.334 "

0.340 "

Lab. No. 627. Diabetic urine, 1710 cc., sp. gr. 1.025. Determinations made as described on p. 220.

Total acetone + diacetic = 0.81 gm. acetone.

Total  $\beta$ -oxybutyric = 2.00 gms. acetone or 3.60 gms.  $\beta$ -oxybutyric acid.

If 250 cc. of this urine (= 0.525 gm.  $\beta$ -oxybutyric acid) were used for a determination by any of the ether extraction methods, the ether residue dissolved in 50 cc. water and the resulting 1.05 per cent solution read in a 200 mm. tube in the polariscope, an error of  $0.05^\circ$  in reading would be 9.5 per cent of the total.

I am indebted to Mr. E. A. Reinoso for carrying out many of these experiments.



# THE INFLUENCE OF COMPLETE THYROIDECTOMY AND OF THYROID FEEDING UPON CERTAIN PHASES OF INTERMEDIARY METABOLISM.

BY FRANK P. UNDERHILL AND TADASU SAIKI

(From the Sheffield Laboratory of Physiological Chemistry, Yale University.)

(Received for publication, July 10, 1908.)

Despite the overwhelming mass of literature recorded<sup>1</sup> relative to the influence of the thyroid upon the nutritional changes in the animal organism the function of this gland still remains in practical obscurity. Attempts toward a solution of the problem have been made from various viewpoints, such as investigations into the metabolic processes attendant upon pathological conditions of the gland and of the action induced in such cases by the administration of fresh and dried glands and of various commercial preparations. A large number of observations are on record of feeding or injection of the thyroid or its constituents to normal man and animals with subsequent determination of the effects exerted upon the general metabolic activities. Studies have also been carried out for the purpose of ascertaining differences in nutritional processes after removal of the organ or a portion of it in an attempt to correlate gland function with specific symptom of metabolic activity, and finally the endeavor to separate thyroid function from that exercised by the parathyroids is at present a theme of much interest and scientific activity.<sup>2</sup>

The present investigation was undertaken with a view of ascertaining possible changes in the intermediary metabolic processes after complete thyroidectomy and after thyroid feeding. The occurrence of unusual variations in the excretion of one or more urinary nitrogenous constituents might indicate a line of attack

<sup>1</sup> For a review of the literature on thyroid, see von Noorden, *Handbuch der Pathologie des Stoffwechsels*, ii, 1907.

<sup>2</sup> Cf. MacCallum and Voegtlin: *Bulletin of the Johns Hopkins Hospital*, xix, p. 91, 1908.

which followed would possibly render thyroid function less obscure. Accordingly the composition of the urine, that is, the relationship of certain of the nitrogenous constituents, has been determined under the experimental conditions outlined.

#### EXPERIMENTAL.

*Methods.* The methods employed for the analysis of the urine were those commonly in use in this laboratory.<sup>1</sup> The feces were not examined. Sugar determinations were carried out according to the Allihn method and the procedure of Pflüger<sup>2</sup> was followed for the estimation of glycogen. The thyroidectomy experiments were performed upon male dogs and catheterization was not practiced. Inasmuch as only the relationships of the nitrogenous substances were desired exactly twenty-four hour specimens were not essential. Bitches were employed in the feeding experiments and the urine was obtained in twenty-four hour periods by catheterization, care being taken against the possibility of bladder infection. The thyroid preparation fed, desiccated thyroid, was obtained from Armour & Co., and contained 12.75 per cent of nitrogen and 0.12 per cent of iodine. Whenever thyroidectomy was performed the operation was carried out under conditions as aseptic as possible and both the thyroid and parathyroids were presumably completely removed. None of the animals operated upon gave evidence of any disturbance due to the operative technique itself and all wounds healed rapidly.

#### *The Composition of the Urine after Complete Thyroidectomy.*

Aside from the observation<sup>3</sup> that extirpation of the thyroid causes a decrease in nitrogen elimination little investigation has been made concerning protein metabolism under these conditions. A study of the partition of the urinary constituents indicative of the more important lines of nitrogenous metabolism has therefore been carried out, the details of which are to be found in Tables 1, 2, 3, 4 and 5.

<sup>1</sup> Cf. Underhill and Kleiner: *This Journal*, iv, p. 165, 1908.

<sup>2</sup> Pflüger: *Archiv für die gesammte Physiologie*, cxi, p. 307, 1906.

<sup>3</sup> Dutto and Lo Monaco: *Archives italiennes de biologie*, xxiv, p. 196, 1895; Roos: *Zeitschrift für physiologische Chemie*, xxi, p. 19, 1895-96.

TABLE 1. COMPOSITION OF URINE AFTER COMPLETE THYROIDECTOMY.  
Dog 1: Glands removed from dog October 7; weight, 10 kilos.

Date.	Volume.	Specific gravity.	Reaction to litmus.	Total N.	NH <sub>3</sub> -N.	Total creatinin N.	Preformed N.	P	NH <sub>3</sub> -N.	Creatinin N.	REMARKS.
	cc.	10-36		gram.	gram.	gram.	gram.	gram.	per cent.	per cent.	
1907 Oct. 8	225	36	alk.	8.46	0.53	0.15	0.13		6.2	1.5	Urine gave intense indican and diacetic acid tests.
9	135	34	"	5.30	0.52	0.09	0.08		9.8	1.6	Milk was drunk greedily but immediately vomited; urine contained protein and gave above tests.
10	175	45	acid	8.01	0.69	0.13	0.12		8.6	1.5	Urine gave reactions noted above.
11	160	27	"	15.62	1.03	0.25	0.25		6.6	1.6	Involuntary twitchings of muscles of mouth, neck and feet. Drank 500 cc. water.
12	240	34	"					0.48	5.5	1.5	Animal's condition practically unchanged.
13 & 14	160	43	"	7.96	0.44	0.15	0.12		8.5	2.0	Drank 30 cc. water.
15	200	33	"	6.00	0.51	0.13	0.12				Urine contained heavy deposit of urates.
											Tests for protein, indican and diacetic acid positive.
16	110	42	"	4.41	0.36	0.11	0.09		8.1	2.0	Animal apparently deaf and blind. Ky-nurenic acid in urine, 0.04 gm.
17	135	35	"	3.82	0.37	0.11	0.10	0.25	9.6	2.6	Dog found dead in cage. Autopsy revealed nothing abnormal.

TABLE 2. COMPOSITION OF URINE AFTER COMPLETE THYROIDECTOMY.

*Dog 2: Glands removed from dog of 9 kilos, October 21.*

Date.	Volume.	Specific gravity.	Reaction to litmus.	Total N.	NH <sub>3</sub> -N.	Total creatinin N.	Preformed creatinin N.	Creatin N.	NH <sub>3</sub> -N.	Total creatinin N.	Preformed creatinin N.	Creatin N.	REMARKS.
1907.	cc.	10-		gram.	gram.	gram.	gram.	gram.	per cent.	per cent.	per cent.	per cent.	
Oct. 22	397	23	alk.	3.94	0.40	0.12	0.10	0.02	10.1	3.0	2.5	0.5	Urine contained sugar.
23	210	36	acid	5.08	0.45	0.15	0.10	0.05	8.8	3.1	2.0	1.1	Urine contained no sugar.
24	167	45	"	7.61	0.67	0.23	0.15	0.08	8.8	3.0	2.0	1.0	Animal did not have control of muscles. Drank 200 cc. milk.
25	162	47	"	7.92	0.57	0.15	0.12	0.03	7.1	1.9	1.5	0.4	Dog in poor condition, respiration labored. Found dead in the evening.

TABLE 3. COMPOSITION OF URINE AFTER COMPLETE THYROIDECTOMY.  
*Dog 3: Glands were removed from a dog of 5 kilos, October 28.*

Date.	Volume.	Specific gravity.	Reaction to litmus.	Total N.	NH <sub>4</sub> -N.	Total creatinin N.	Preformed creatinin N.	Creatin N.	NH <sub>3</sub> -N.	Total creatinin N.	Preformed creatinin N.	Creatin N.	REMARKS.
	cc.	10°		gram.	gram.	gram.	gram.	gram.	per cent.	per cent.	per cent.	per cent.	
1907													
Oct. 29	145	43	amph.	5.44	0.39	0.13	0.08	0.05	7.1	2.4	1.4	1.0	Urine gave protein and di-acetic acid tests.
30	98	46	acid	4.56	0.40	0.12	0.07	0.05	8.7	2.6	1.5	1.1	Drank 270 cc. milk.
31	85	51	"	3.90	0.34	0.10	0.06	0.04	8.7	2.5	1.5	1.0	Tetany, dyspnea. Diet—30 grams very dry bread and 300 cc. milk.
Nov. 1	68	51	"	3.02	0.26	0.05	0.05	0.00	8.6	1.6	1.6	0.0	Condition unchanged. Diet—30 grams very dry bread and 300 cc. milk.
4	126	43	"	4.12	0.42	0.10	0.09	0.01	10.2	2.4	2.1	0.3	Food refused. Urine gave strong diacetic acid test.
5	120	40	"	3.20	0.33	0.09	0.09	0.00	10.3	2.8	2.8	0.0	"

TABLE 4. COMPOSITION OF URINE AFTER COMPLETE THYROIDECTOMY.  
Dog 10: Glands were removed from a dog of 14 kilos, March 23.

Date.	Volume.	Specific gravity.	Reaction to litmus.	Total N.	Purin N.	Allantoin N.	Uric acid N.	Purin N.	Allantoin N.	Uric acid N.	REMARKS.
1908.	cc.	10—		gram.	gram.	gram.	gram.	per cent.	per cent.	per cent.	
Mar. 24	182	52	alk.	6.88	0.145	0.023		2.1	0.3		
25	63	52	acid	3.51		0.058			1.6		
27	128	59	"	6.21	0.023	0.021	0.024	0.3	0.2	0.3	Diet = 120 cc. milk + 200 cc. water. Urine contained crystals of allantoin. Urine of March 26 was discarded since it contained vomited material.
28	85	30	"	2.29	0.021	trace	trace	0.9			
29	133	47	"	5.41	0.025	"	0.007	0.4		0.1	
30	80	48	"	3.60	0.015	"		0.4			
31	90	53	"	4.79	0.013	"	trace	0.2			Animal in very poor condition. Killed.

Dog 11: Glands removed from dog of 15.8 kilos, March 23.

Mar. 24	168	53	alk.	6.57	0.158	0.011	0.011	2.4	0.1	0.2	Drank 300 cc. water. Dyspnoea.
25	145	40	acid								
26	105	50	"	10.03	0.155	0.032	0.034	1.5	0.3	0.3	Dyspnoea has disappeared.
27	183	53	"	8.83	0.092	0.100	0.011	1.0	1.1	0.1	Animal in very poor condition. Killed.



TABLE 5. DISTRIBUTION OF URINARY SULPHUR AFTER COMPLETE THYROIDECTOMY.

Date.	Total S.	Inorganic S.	Ethereal S.	Neutral S.	Inorganic S.	Ethereal S.	Neutral S.	REMARKS.
1907.	gram. (0.638)	gram. (0.333)	gram. (0.045)	gram. (0.260)	per cent.	per cent.	per cent.	These figures were obtained from urine of animal employed in Table 2. Figures in parentheses are results obtained on a composite of two days' urine.
Oct. 23 & 24	0.319	0.166	0.022	0.130	52.0	6.9	40.8	
25	0.314	0.192	0.024	0.098	61.1	7.6	31.2	
Nov.	0.185	0.087	0.017	0.081	47.0	9.1	47.0	From same animal as above. These figures were obtained from urine of Dog 3, given in Table 3.

The most significant change apparent in the distribution of the urinary substances determined is the increased ratio of nitrogen in the form of ammonia. This cannot be entirely accounted for by the condition of partial or complete inanition since the increase is greater than occurs in the latter conditions.<sup>1</sup> Moreover, feeding does not affect this ratio as may be seen from Table 3 where the percentage of ammonia is as high after the intake of food as it is in Table 1 where the animal was fasting. It is therefore probable that a corresponding decrease in the output of urea occurred. Whether any relationship exists between the increased output of ammonia and the practically constant positive test for diacetic acid was not determined. An interesting feature with respect to the extirpation of the glands in the present experiments is the almost invariable alkalinity of the urine for a few days following the operation. While this work was in progress a paper appeared by Coronedi and Luzzatto<sup>2</sup> in which it was shown that the urine is alkaline after thyroidectomy owing to an increased output of ammonia. These conditions were entirely independent of diet and were not due to infection from catheterization. Our own experiments confirm those of Coronedi and Luzzatto with regard to an increased output of ammonia but differ in that the urine remains alkaline only for a day or two although it must be admitted that the acid reaction is never as strong after thyroidectomy as that usually found associated with dog's urine.

The total creatinin excretion is practically the same in the three animals examined although Dog 1 weighed twice as much as Dog 3. Whereas the percentage of nitrogen in the form of creatinin is fairly constant, that existing as creatin varies widely; for example, the urine of Dog 1 contains little or no creatin nitrogen while in that of Dog 2 creatin nitrogen is present at times to the extent of 1 per cent. The elimination of nitrogen as purin bodies and allantoin is about the same as the excretion of these substances noted in the urine of a fasting dog. The few phosphorus determinations made show a small output, possibly indicating,

<sup>1</sup> Oesterberg and Wolf: *Biochemische Zeitschrift*, v, p. 304, 1907; Underhill and Kleiner: *loc. cit.*, and also cf. Richards and Wallace: *This Journal*, iv, p. 179, 1908.

<sup>2</sup> Coronedi and Luzzatto: *Archives italiennes de biologie*, xlvii, p. 286, 1907.

when considered with the low purin and allantoin excretion, a low rate of nuclear disintegration. Sulphur partition in the urine gives evidences of little change from the normal although the number of estimations made are too few to draw definite conclusions. According to Ducceschi<sup>1</sup> the total and neutral sulphur of the urine is increased after thyroidectomy which has been interpreted as significant of a lowered rate of oxidation.

In cachexia thyreopriva<sup>2</sup> pathological changes have been observed in certain of the nervous structures and Vassale and Donaggio<sup>3</sup> noted degeneration of the pyramidal tract following thyroidectomy in dogs. Attendant upon certain acute degenerative diseases of the nervous system Rosenheim<sup>4</sup> has shown the presence of significant quantities of cholin in the blood, cerebrospinal system and tissues. The possibility therefore suggested itself that if thyroidectomy were responsible for degeneration of nervous structures the presence of cholin in the blood could be taken as evidence of disturbed nervous metabolism. To test this possibility two animals were allowed to live after thyroid extirpation until death seemed imminent when they were chloroformed and blood withdrawn from the carotid. Cholin was tested for by the method outlined by Rosenheim<sup>5</sup> but the results were negative.

#### *Certain Aspects of Carbohydrate Metabolism after Complete Thyroidectomy.*

A former communication<sup>6</sup> from this laboratory corroborated Scott's<sup>7</sup> demonstration of the ability of the animal body to utilize large quantities of dextrose subcutaneously introduced. After doses varying from 5 to 7 grams of dextrose per kilo of body weight injected hypodermically into dogs only traces reappear in the urine and then only for the first day or two subsequent to

<sup>1</sup> Ducceschi: *Ibid.*, xxvi, p. 209, 1896.

<sup>2</sup> Schäfer: *Text Book of Physiology*, i, p. 941.

<sup>3</sup> Vassale and Donaggio: *Archives italiennes de biologie*, xxvii, p. 129, 1896.

<sup>4</sup> Rosenheim: *Journal of Physiology*, xxxv, p. 465, 1906-07.

<sup>5</sup> Rosenheim: *Ibid.*, xxxiii, p. 220, 1905-06.

<sup>6</sup> Underhill and Closson: *This Journal*, ii, p. 117, 1906.

<sup>7</sup> Scott: *Journal of Physiology*, xxxiii, p. 107, 1902.

the injection. Comparable experiments (see Table 6) have been carried out upon two dogs after complete thyroidectomy.

The results obtained under these experimental conditions, however, present an entirely different degree of utilization of the sugar introduced. Under normal circumstances little more than a trace of the dextrose injected is eliminated in the urine, whereas with Dog 3 almost one-half the quantity introduced reappeared before the animal died. That this did not represent the entire quantity which would have been excreted had the animal lived longer is shown by the observation that the urine found in the bladder after death possessed strong reducing powers. These results would appear therefore to indicate a diminished ability on the part of the body to utilize carbohydrate material, at least when subcutaneously introduced. The lack of utilization may be regarded as a lessened power of oxidation or glycolysis, or as a decreased ability to transform dextrose into glycogen.

An experiment was performed to test the latter hypothesis, the details of which follow: A dog of 10.2 kilos was allowed to fast for one week and then complete thyroidectomy was performed. On the two following days 40 grams of dextrose (80 grams in all) were injected subcutaneously. The animal was killed next day with chloroform and glycogen determinations were made on the liver. The urine contained 1.6 gram of dextrose. The liver weighed 261 grams and contained 0.43 gram glycogen. A control experiment with a normal dog, after a like period of inanition and a similar injection of dextrose yielded 0.64 gram glycogen in a liver weighing 270 grams. The quantities of glycogen obtained in the two cases force one to the conclusion that complete thyroidectomy does not appreciably reduce the glycogenic function of the animal body. A lessened oxidative or glycolytic activity subsequent to complete extirpation of the thyroids would therefore appear to be indicated. This assumption is in harmony with the observations of Magnus-Levy<sup>1</sup> who has shown that the respiratory quotient is low in myxœdema and cretinism, and is high in Basedow's disease and after feeding thyroid preparations to normal man.

<sup>1</sup> Magnus-Levy: *Zeitschrift für klinische Medizin*, xxxiii, p. 269, 1897, and lx, p. 177, 1906.

TABLE 6. COMPOSITION OF URINE AFTER COMPLETE THYROIDECTOMY AND SUBCUTANEOUS INJECTION OF DEXTROSE.  
*Dog 3: Same dog as in Table 5.*

Date.	Volume.	Specific gravity.	Reaction to litmus.	Total N.	NH <sub>4</sub> -N.	NH <sub>4</sub> -N.	Dextrose.	REMARKS.
	cc.	10—		gram.	gram.	per cent.	gram.	
1907. Nov. 5	120	40	acid	3.20	0.33	10.3		This experiment is a continuation of that outlined in Table 3.
6	94	44	"	3.04	0.28	9.2		Subcutaneous injection of 5 grams dextrose per kilo in 20 per cent solution.
7	82	53	"	2.84	0.31	10.9	2.48	Urine contained protein and gave strong diacetic acid test; after fermentation urine gave no reduction and was optically inactive.
8	79	53	"	3.28	0.31	9.4	1.92	Animal unable to retain food (bread and milk). Urine optically active after fermentation — 0.7° in 100 mm.
9	82	49	"	3.09	0.30	9.7	1.45	Urine contained protein.
10 & 11	150	48	"	5.54	0.30	5.4	3.46	" "
12	82		"	1.84	0.17	9.2	1.25	Dog found dead. Urine in bladder gave strong reduction.

*Dog 4: Glands removed from dog, November 21, weight = 6.4 kilos.*

Nov. 22	160		acid	5.57	0.13	2.3		Subcutaneous injection of 5 grams dextrose per kilo in 30 per cent solution.
23	179	41	"	6.34	0.21	3.3	1.49	Animal drank 50 cc. water. After fermentation urine was optically inactive.
25	116	43	"	3.59	0.20	5.5	1.51	Animal drank water. No urine for Nov. 24.
26	115	33	"	2.99	0.16	5.3	0.19	Dog found dead in cage. Urine in bladder gave strong reduction.

*The Composition of the Urine of Normal Dogs after Thyroid Feeding.*

As a consequence of the large number of investigations<sup>1</sup> that have been carried out with thyroid feeding both with normal man and animals the idea has gradually been evolved that the thyroid gland acts as a stimulus to protein catabolism. In experiments with animals Roos<sup>2</sup> and Voit<sup>3</sup> in particular have been largely responsible for the theory as to this mode of action of the thyroid on metabolism. In their investigations it was impossible to maintain animals in nitrogenous equilibrium, more nitrogen being eliminated than was introduced. Schöndorff<sup>4</sup> obtained similar results but offered the explanation that the increased nitrogen output was due to an increased excretion of urea and other extrac-tives present in the body fluids. According to this author pro-tein in the body is not attacked if sufficient fat is present. The more recent work of Schryver<sup>5</sup> demonstrating the influence of thyroid feeding upon autolytic processes in the liver would tend to corroborate the view that thyroid feeding has a stimulating action upon catabolic processes although Wells<sup>6</sup> failed to get evidence of such an action when thyroid extract was directly added to liver pulp.

The experiments here recorded were made with two bitches fed upon a standard diet composed of meat, cracker meal and lard with a definite volume of water. The animals received this diet several days previous to the beginning of the experiment and were practically in nitrogenous equilibrium. Different quantities of thyroid were added to the standard diet at intervals and the influ-ence of this administration upon the composition of the urine is shown in Tables 7 and 8.

From these tables it is readily seen that urinary nitrogen excre-tion is but little influenced by thyroid feeding. When thyroid

<sup>1</sup> A review of the literature is given by Andersson and Bergmann: *Skandinavisches Archiv für Physiologie* viii, p. 326, 1897-98. The literature is brought up to date by von Noorden, *loc. cit.*

<sup>2</sup> Roos: *Zeitschrift für physiologische Chemie*, xxi, p. 19, 1895-96; xxv, p. 1, and p. 242, 1898; xxvii, p. 40, 1899.

<sup>3</sup> Voit: *Zeitschrift für Biologie*, xxxv, p. 116, 1897.

<sup>4</sup> Schöndorff: *Archiv für die gesammte Physiologie*, lxvii, p. 395, 1897.

<sup>5</sup> Schryver: *Journal of Physiology*, xxxii, p. 159, 1905.

<sup>6</sup> Wells: *Americal Journal of Physiology*, xi, p. 351, 1904.

TABLE 7. COMPOSITION OF URINE AFTER THYROID FEEDING.

Dog 9.

Date.	Volume.	Specific gravity.	Reaction to litmus.	Total N.	NH <sub>3</sub> -N.	Purin N.	Allantoin N.	Total creatinin N.	Preformed creatinin N.	Creatin N.	P.	NH <sub>3</sub> -N.	Purin N.	Allantoin N.	Total creatinin N.	Preformed creatinin N.	Creatin N.	REMARKS.
1908.	cc. 10-52		acid	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	p. c. p. c. p. c. p. c. p. c.	p. c. p. c. p. c. p. c. p. c.	p. c. p. c. p. c. p. c. p. c.	p. c. p. c. p. c. p. c. p. c.	p. c. p. c. p. c. p. c. p. c.	p. c. p. c. p. c. p. c. p. c.	
Feb. 10	105	52		5.260	19.0	0.096	0.100	0.210	0.080	0.130	0.36	3.61	8.1	9.4	0.1	5.2	5	Body weight = 8.0 kilos. Standard diet. N in food = 6.0 gms.
11	114	52	"	5.330	18.0	0.034	0.272	0.210	0.090	0.120	0.36	3.30	6.5	1.3	9.1	0.2	3	"
12	95	62	"	5.850	21.0	0.032	0.144	0.230	0.080	0.150	0.42	3.50	5.2	4.3	9.1	0.2	6	Standard Diet + 5 gms thyroid. N in food = 6.6 gms.
13	115	59	"	6.890	26.0	0.018	0.189	0.230	0.100	0.130	0.36	3.70	3.2	9.3	3.1	4.1	9	"
14	140	52	"	7.270	23.0	0.055	0.024	0.260	0.080	0.180	0.36	3.10	7.0	3.3	5.1	1.2	4	"
15	121	56	"	6.480	20.0	0.010	0.080	0.240	0.070	0.170	0.33	3.00	1.1	2.3	7.1	0.2	6	"
16	115	60	"	6.730	25.0	0.016	0.352	0.250	0.070	0.180	0.33	3.70	2.5	2.3	7.1	0.2	6	"
17	132	54	"	6.890	26.0	0.032	0.436	0.260	0.080	0.180	0.36	3.70	4.6	1.3	7.1	1.2	6	"
18	140	45	"	6.860	26.0	0.106	0.247	0.270	0.070	0.200	0.36	3.70	1.3	6.3	9.1	0.2	9	"
19	110	53	"	6.320	23.0	0.094	0.121	0.230	0.070	0.180	0.27	3.61	4.1	9.3	6.1	1.2	5	Standard diet. N in food = 6.0 gms.
20	105	54	"	5.530	23.0	0.016	0.030	0.230	0.080	0.170	0.27	4.10	2.0	5.4	1.1	4.2	6	"
21	85	59	"	5.150	21.0	0.020	0.110	0.200	0.080	0.120	0.24	4.00	3.2	1.3	9.1	1.5	2.4	"
22	87	60	"	5.510	22.0	0.012	0.048	0.190	0.070	0.150	0.27	3.90	2.0	8.3	4.1	2.2	2	"
23	105	50	"	5.200	23.0	0.104	0.062	0.180	0.080	0.100	0.28	4.42	0.1	2.3	4.1	1.5	1	"
24	188	34	"	5.420	24.0	0.120	0.048	0.200	0.080	0.120	0.30	4.42	0.0	9.3	7.1	4.2	3	Standard diet + 20 gms. thyroid. + 10 cc. H <sub>2</sub> O (extra). N in food = 8.5 gms.
25	260	35	"	7.630	32.0	0.040	0.168	0.310	0.090	0.160	0.36	4.10	5.2	2.4	0.1	1.2	9	"
26	195	44	"	8.330	32.0	0.040	0.192	0.270	0.080	0.190	0.33	3.80	4.2	3.3	2.0	9.2	3	"

TABLE 7—CONTINUED.

Date	Volume.	Specific gravity.	Reaction to litmus.	Total N.	NH <sub>3</sub> -N.	Purin N.	Allantoin N.	Total creatinin N.	Preformed creatinin N.	Creatinin N.	P.	NH <sub>3</sub> -N.	Purin N.	Allantoin N.	Total creatinin N.	Preformed creatinin N.	Creatinin N.	REMARKS.
1903	cc.	10—		gm.	gm.	gm.	gm.	gm.	gm.	mg.	gm.	p. c.	p. c.	p. c.	p. c.	p. c.	p. c.	
Feb. 27	152	52	acid	8.10	0.27	0.040		0.27	0.07	0.20	0.27	3.30	3.30	5	3.30	3.82	5	Standard diet. N in food = 6.0 gms.
28	115	53	"	5.60	0.22	0.024	0.056	0.23	0.08	0.17	0.24	3.90	3.90	4	1.04	1.14	2.7	"
29	100	50	"	4.76	0.17		0.048	0.16	0.07	0.09	0.25	3.5	3.5	1	0.33	1.41	9	"
Nov. 1	95	50	"	4.61	0.17	0.044	0.016	0.16	0.07	0.09	0.21	3.6	3.6	0	3.34	1.51	9	"
2	82	53	"	4.59	0.20	0.026	0.128	0.16	0.07	0.09	0.24	4.3	4.3	0	5.28	3.51	52	Standard diet + 50 gms thyroid + 10 cc. water (extra). N in food = 12.33 gms.
3	164	62	"	9.36	0.35	0.064	0.215	0.26	0.10	0.16	0.39	3.7	3.7	0	7.22	2.71	0	Standard diet. N in food = 6.0 gms.
4	132	49	"	6.71	0.30	0.032	0.088	0.26	0.09	0.17	0.27	4.4	4.4	0	4.13	3.81	3	"
5	123	50	"	6.07	0.21	0.030	0.256	0.26	0.10	0.16	0.24	3.4	3.4	0	5.42	4.21	6	"
6	85	53	"	5.06	0.21	0.035	0.140	0.16	0.08	0.08	0.24	4.1	4.1	0	7.27	3.11	5	"
7	94	58	"	5.38	0.20	0.026	0.192	0.20	0.09	0.11	0.24	3.7	3.7	0	4.35	3.71	7	Standard diet. N in food = 6.0 gms. Body weight = 7.3 kilos.



TABLE 8. COMPOSITION OF URINE AFTER THYROID FEEDING.  
Dog 8

Date.	Volume.	Specific Gravity.	Reaction to litmus.	Total N.	NH <sub>3</sub> -N.	Purin N.	Allantoin N.	Total creatinin N.	Performed creatinin N.	Creatinin N.	P	NH <sub>3</sub> -N.	Purin N.	Allantoin N.	Total creatinin N.	Performed creatinin N.	Creatinin N.	REMARKS.
1903.	cc.	10°	f'dly acid	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	p. c.	p. c.	p. c.	p. c.	p. c.	p. c.	
Jun. 14	232	45	"	11.14	0.42	0.022	traces	0.27	0.22	0.05		3.70	0.2		2.41	0.05		Standard diet. N in food = 9.6 gm.
15	265	39	"	9.53	0.39	0.026	"	0.28	0.19	0.09		4.00	0.2		2.91	0.10	"	"
16	200	45	"	8.09	0.33	0.026	"	0.24	0.17	0.07	0.95	4.00	0.3		2.92	0.08		30 gms. thyroid added to diet + 50 cc. water. Entire food not eaten. N in food = 12.4 gms.
17	430	31	"	12.78	0.76	0.089	0.086	0.37	0.19	0.18	1.02	5.90	0.7	0.7	2.91	0.15		Standard diet. N in food = 9.6 gm.
18	250	36	amp.	9.43	0.66	0.040	0.070	0.34	0.17	0.17	1.72	6.90	0.4	0.7	3.61	0.18		"
21 <sup>10</sup>	480	50	"	10.51	0.52	0.020	0.010	0.50	0.30	0.20	1.78	3.10	0.1	0.06	3.01	0.12		"
21	115	50	acid	6.96	0.31	0.096	0.090	0.19	0.16	0.03	0.57	4.41	0.3	1.2	2.72	0.05		"
22	188	43	"	7.51	0.35	0.144	0.040	0.30	0.16	0.17		4.61	0.9	0.5	3.92	0.18		"
3	155	40	"	6.17	0.31	0.096	0.081	0.14	0.13	0.01	0.38	5.01	0.5	1.0	2.22	0.10		30 gms. thyroid + 50 cc. H <sub>2</sub> O added to diet. N in food = 13.4 gm.
21	369	35	"	12.48	0.65	0.220	0.828	0.30	0.22	0.08	0.61	5.21	0.6	0.6	2.41	0.06		"
25	295	45	"	12.19	0.49	0.454	0.136	0.22	0.14	0.08	0.60	4.03	0.7	1.1	1.81	0.06		"
26	225	49	"	9.96	0.38	0.378	0.856	0.27	0.16	0.11	0.44	3.83	0.8	0.5	2.71	0.11		Standard diet. N in food = 9.6 gm.
27	275	40	"	9.91	0.29	0.360	0.130	0.29	0.17	0.15	0.56	2.93	0.6	1.3	2.91	0.12		Standard diet. N in food = 4.8 gm. Portion of diet uneaten.
28	180	44	"	5.95	0.20	0.168	0.076	0.20	0.14	0.06	0.37	3.32	0.8	1.2	3.32	0.10		Standard diet. N in food = about 4.0 gm. Portion of diet uneaten.

is first fed the nitrogen excretion in the urine may exceed the quantity introduced into the body but this influence is apparently soon lost on cessation of thyroid administration. A small dose (5 grams) appears to exert as great an influence upon nitrogen excretion as a much larger quantity.

Unlike the results obtained with thyroidectomized animals no change occurs in the elimination of ammonia nitrogen. With small doses of thyroid the output of purin and allantoin nitrogen is variable but after continued large doses the quantity of purin nitrogen is largely increased. Allantoin nitrogen is extremely variable and appears to bear little relation to thyroid intake or purin excretion. The phosphorus excretion is low. The elimination of creatinin is scarcely affected by thyroid feeding. Creatin was a constant constituent of the urine of both dogs. Indeed in Dog 9, Table 7, the nitrogen existing in this form is in excess of that in the form of creatinin. Reducing substances were never present in the urine. Thyroid feeding, therefore, whether in small or large doses, single or repeated has little influence in changing the ratios normally existing between the urinary nitrogenous constituents. Our experiences with thyroid feeding impresses us that the action of this gland on protein catabolism should not be over-emphasized, but that more attention should be directed toward ascertaining the influence of the thyroid on the metabolism of the carbohydrates and fats, and especially its relation to various inorganic<sup>1</sup> constituents of the body.

#### SUMMARY.

After complete thyroidectomy (and parathyroidectomy?) in dogs *the ammonia output in the urine is increased*, even beyond what is observed in starving animals.

The urinary nitrogen eliminated as creatinin, purin bodies and allantoin is about equal in amount to what has been noted in fasting dogs.

As in normal fasting dogs a variable and frequently not inconsiderable output of creatin was observed.

Thyroidectomized dogs are *incapable of utilizing subcutaneously introduced dextrose* in anywhere near the same degree as

<sup>1</sup> MacCallum and Voegtlin: *loc. cit.*

normal animals. It is thus suggested that the loss of the glands may occasion a change in the gaseous metabolism similar to what has been observed in myxœdema, cretinism, etc.

Thyroid tissue fed to normal dogs causes a slight increase in urinary nitrogen excretion. This influence soon disappears on cessation of thyroid administration. Small doses of thyroid appear to have as pronounced an influence upon the nitrogen elimination as larger ones.

A larger output of purin-nitrogen and a low output of phosphorus were observed after the continued administration of large doses of thyroid tissue.

*Thyroid feeding produces little change in the interrelation of the nitrogenous urinary constituents.* This is in harmony with the accumulating evidence that these relationships are upset only by exceptionally profound disturbances in the nutrition of the cells.<sup>1</sup>

<sup>1</sup> Jackson and Pearce: *Journal of Experimental Medicine*, ix, p. 552, 1907; Underhill and Kleiner: *loc. cit.*; Richards and Wallace: *loc. cit.*



# ON A GLOBULIN FROM THE EGG YOLK OF THE SPINY DOG-FISH, *SQUALUS ACANTHIAS* L.

By C. L. ALSBERG AND E. D. CLARK.

(From the United States Fisheries Laboratory at Woods Hole, and the Department of Biological Chemistry of the Harvard Medical School.)

(Received for publication, July 6, 1908.)

The yolk of the hen's egg contains a great deal of an iron-containing phosphoprotein with the physical properties of a globulin, known as ovovitellin. It has been repeatedly made the subject of investigation.<sup>1</sup> The only other vitellins that have been studied were obtained from the eggs of teleost fishes. A study of the vitellins from other classes of animals seems desirable because it might conceivably throw light upon some of the processes which go on in the yolk during development. It seems that vitellin has the important functions of furnishing iron<sup>2</sup> and pyrrol<sup>3</sup> for the hæmoglobin formation, as well as some of the phosphorus needed for growth. Even among the vitellins of a single class, the teleosts, there seem to be considerable differences. The vitellin of the carp's egg is a glycoprotein yielding a reducing substance on hydrolysis. It also contains phosphorus and iron.<sup>4</sup> The vitellins obtained by Levene<sup>5</sup> from cod-fish eggs and by Hammarsten<sup>6</sup> from perch eggs did not yield a reducing substance.

It is therefore not without interest to investigate the egg of a selachian as to its vitellin, particularly one that is viviparous as is *Squalus acanthias*. It is conceivable that the carrying of the eggs by the female may affect the composition of the yolk even when there is no true placentation.

<sup>1</sup> Cf. Hammarsten: *Text-Book*, pp. 503-504, 1908.

<sup>2</sup> Bunge: *Zeitschr. f. physiol. Chem.*, ix, p. 49.

<sup>3</sup> Levene and Alsberg: *This Journal*, ii, p. 133.

<sup>4</sup> Walter: *Zeitschr. f. physiol. Chem.*, xv.

<sup>5</sup> Levene: *Ibid.*, xxxii.

<sup>6</sup> Hammarsten: *Skand. Arch. j. Physiol.*, xvii.

## 244 Globulin from Egg Yolk of *Squalus Acanthias*

The eggs of this species are quite round and about 2.5 cm. in diameter. The membrane is elastic and when it is ruptured the yolk flows out as a slightly yellowish cream much thinner than the yolk of the hen's egg. The yolk was mixed with an equal volume of 10 per cent sodium chloride solution and extracted with ether in a separatory funnel until but little fat passed into the ether.<sup>1</sup> It was then precipitated by dilution with water and the precipitate allowed to settle. In a few hours the water was siphoned off and the white precipitate centrifugated from the remainder of the solution. Centrifugation is much to be preferred to filtration in dealing with vitellins, for the time consumed is much shortened and the loss as the result of the substance becoming insoluble upon the filter much diminished. The precipitate is then redissolved in 10 per cent sodium chloride and filtered. The filtrate is reprecipitated by dilution and washed by decantation with ether-water until the latter is free from chlorides and biuret giving substances. It is then collected on a filter, washed with alcohol, transferred to a boiling flask with reflux condenser and boiled out with alcohol many times. Only slowly and after a long time is all the alcohol-soluble material removed. It is then boiled out with ether, and dried *in vacuo* over sulphuric acid at 70° C. The yield from a relatively small amount of yolk was very considerable.

The material thus obtained is a more or less yellowish or brownish white powder giving the biuret reaction and a distinct, though weak, Hopkins-Cole reaction for tryptophan. Millon's reaction is exceedingly weak or possibly negative. It differs therefore markedly as regards the protein color reactions from vitellin. The orcin and Molisch reactions are negative. On hydrolysis with mineral acid it does not yield a reducing substance, resembling in this respect the majority of the vitellins. On boiling with alkaline lead solution it gives a very faint blackening of the solution, though the material itself as it passes into solution turns black. The fresh material is soluble in dilute salt solutions, insoluble in water. Its precipitation limits as well as its coagulation temperature were not determined. We have therefore no guarantee that we are not determined. We mixture of two or more globulins.

<sup>1</sup> Levene and Alsberg: *Zeitschr. f. physiol. Chem.*, xxi.

On analysis it yields the following results:

PREPARATION I.

0.2117 gm. gave 3.40 cc. dry N at 768 mm. and  $21^{\circ}$ : N = 16.85 per cent.  
0.2280 gm. gave 0.4240 gm.  $\text{CO}_2$  and 0.1450 gm.  $\text{H}_2\text{O}$ : C = 50.72 per cent.  
H = 7.12 per cent.

PREPARATION II.

0.1756 gm. gave 25.05 cc. dry N at 758 mm. and  $20^{\circ}$ : N = 16.58 per cent.  
0.1884 gm. gave 0.3471 gm.  $\text{CO}_2$  and 0.1219 gm.  $\text{H}_2\text{O}$ : C = 50.25 per cent.  
H = 7.24 per cent.  
0.5515 gm. gave 0.0388 gm.  $\text{BaSO}_4$ : S = 0.914 per cent.

(Determination by fusion with sodium peroxide according to Folin.)

PREPARATION III.

0.1965 gm. gave 27.90 cc. dry N at 772 mm. and  $19^{\circ}$ : N = 16.86 per cent.  
0.1886 gm. gave 0.1200 gm.  $\text{H}_2\text{O}$ : H = 7.12 per cent ( $\text{CO}_2$  determination was lost).  
0.2143 gm. gave 0.0032 gm. ash: Ash = 1.88 per cent.

As only one ash determination was done, none of the above analyses are calculated for the ash-free substance. The ashing was done in platinum and the ash was a fusible one of a slightly brownish color. Treated with hot hydrochloric acid it did not apparently dissolve and in the hydrochloric acid extract no phosphorus and only the merest trace of iron (potassium sulphocyanide) could be discovered. To confirm the absence of phosphorus the substance was repeatedly decomposed by boiling with concentrated nitric acid and this element searched for among the decomposition products. Finally 0.2 gram were fused in silver with sodium hydroxide and potassium nitrate, the fusion mass dissolved, acidified with nitric acid, enough ammonium nitrate added to make 15 per cent and ammonium molybdate added. Not a trace of ammonium phosphomolybdate precipitated though the solution was finally concentrated and stood for many days. Plainly phosphorus is absent in our preparations. The iron content, judging by the qualitative tests, is so exceedingly slight that it is doubtful whether it is really a component or an accidental contamination.

To confirm the absence of phosphorus and perhaps clear up the question as to the occurrence of iron, an attempt was made to prepare hæmatogen, in which the iron and phosphorus if present

## 246 Globulin from Egg Yolk of *Squalus Acanthias*

ought to be concentrated. About 4 grams of the material were suspended in 200 cc. of artificial gastric juice and digested at 40° C. for 40 hours. The material went almost completely into solution and at the end of the digestion only an exceedingly small amount of sediment was present. Further digestion did not increase it. It was collected on an ash-free filter washed with water and alcohol and ashed. There was less than 10 mg. of ash which was completely soluble in hydrochloric acid but contained no phosphorus. It did, however, contain a little iron, in fact considerably more than the original material. Still even here the amount was very small. Plainly our globulin differs from vitellin in not yielding a phosphorus-rich hæmatogen, and it is doubtful whether it contains iron in its molecule.

As all our preparations were made from a single batch of eggs, we can not be sure that some disturbing factors did not affect our preparations; however, this seems very improbable. Almost certainly the eggs of *Squalus acanthias* contain no vitellin in the strict sense of the word; but in its place a globulin or a mixture of them, containing no phosphorus and perhaps no iron. It is, so far as we are aware, the only vertebrate egg in which this has been found to be the case. It would be interesting to investigate whether there is any relation to the fact that *Acanthias* is viviparous. This might easily be done by comparison with other viviparous and oviparous species of selachians. It would also be interesting to see whether the *acanthias* egg contains any considerable amount of iron, and if so, in what form. A similar investigation of the phosphorus would also be worth while.



# CHEMICAL EVIDENCE OF PEPTONIZATION IN RAW AND PASTEURIZED MILK.

BY RACHEL H. COLWELL AND H. C. SHERMAN.

(Contributions from the Havemeyer Laboratories of Columbia University,  
No. 157.)

(Received for publication, July 13, 1908.)

It is well-known that milk which has been heated to a sufficient temperature to destroy most of the acid-forming bacteria may still contain putrefactive or "peptonizing" organisms and it has been suggested that this may constitute an objection to the pasteurization of milk as a preservative measure since the latter forms may grow more readily after destruction of the former and the absence of a sour odor or taste may result in the milk being used as food after objectionable products have been formed by the action of these putrefactive or "peptonizing" bacteria upon the milk proteids. On the other hand, it is held by some bacteriologists that the danger from this source is comparatively unimportant, the common proteolytic bacteria being largely rendered inert by the heating.

In the belief that the bacterial decomposition of proteins in milk might perhaps be measured by means of the ammonia produced, a method for the determination of ammonia was adapted to milk and a somewhat extended study of the development of ammonia in raw, chemically preserved, and pasteurized milk was carried out in this laboratory.<sup>1</sup> In the last and most systematic of these series of experiments, ten samples representing the milk sold by six different dealers were taken at intervals during a period of two months, February to April, 1907. Each of

<sup>1</sup> Berg and Sherman: The Determination of Ammonia in Milk, *Journ. Amer. Chem. Soc.*, xxvii, p. 124; Sherman, Berg, Cohen and Whitman: Ammonia in Milk and its Development during Proteolysis under the Influence of Strong Antiseptics, this *Journal*, iii, p. 171; Whitman and Sherman: The Effect of Pasteurization upon the Development of Ammonia in Milk, *Journ. Amer. Chem. Soc.*, xxx, p. 1288.

these samples was mixed and divided into three portions, one of which was kept raw, one pasteurized at 65° C. and one at 85° C., after which all were brought to a temperature of 15° to 20° C. and examined regularly after standing for two, four and seven days. From the results of these experiments which are given in full elsewhere<sup>1</sup> it appeared that the pasteurization was less efficient in checking the development of ammonia than in checking the production of acid and this was especially true of the milk pasteurized at the higher temperature (85° C.) which before becoming sour often showed an amount of ammonia considerably in excess of that contained in unpasteurized milk of the same age and origin. It was found, however, that the determination of ammonia in milk cannot serve as an exact measure of the extent of protein decomposition in all cases, since in raw milk there was often, and in milk pasteurized at 65° C. there was sometimes, a decrease in the ammonia content between the fourth and the seventh days. In addition to the ammonia determinations each sample was regularly examined as to odor and taste and it was found that the samples kept raw always developed within a few days a sour taste and an odor which was always sour, sometimes "musty" or "cheesy," but never putrid or offensive, while the samples which had been pasteurized, especially at the higher temperature, frequently developed an offensive, putrid odor and a bitter taste. The occurrence of bitter tastes in the milk pasteurized at the higher temperatures, together with the observation that the ammonia content could not serve as a strictly quantitative means of comparing the decomposition of protein in raw and pasteurized milk, suggested the desirability of supplementing the study of the ammonia content by comparative tests for peptone in samples kept raw and after pasteurization at different temperatures.

#### EXPERIMENTAL.

During April and May, 1908, nine samples representing the milk of six retail dealers were studied. Each sample was brought to the laboratory as purchased, mixed and divided (in sterile receptacles with precautions against secondary contamination) into four portions, one of which was kept raw, the others pas-

<sup>1</sup> Whitman and Sherman: *Loc. cit.*

teurized by heating for 20 minutes at 60°, 75° and 90° C., respectively. The pasteurized samples were then cooled and all were allowed to stand at room temperature. After two days, and usually again after four days standing, the odor was observed, the acidity determined, and a test was made for peptone. In testing for peptone, the coagulable protein was first removed, the proteoses, precipitated by zinc sulphate and an aliquot part of the filtrate was treated with sodium hydroxide until the zinc was precipitated and redissolved and was then submitted to the biuret test. The volume of milk tested and the quantities of reagents used having been kept uniform, it is believed that the comparative amounts of peptone present are roughly indicated by the intensity of the color reactions obtained.

The term "acidity" here indicates the number of cubic centimeters of tenth-normal sodium hydroxide required to neutralize 10 cc. of milk using phenolphthalein as indicator.

For convenience in comparing the intensity of the biuret reaction in the different tests, those in which no reaction was obtained are marked 0; those showing a faint reaction, 1; those with distinct reaction, 2; those with strong reaction, 3.

In order to compare the offensiveness of the odor, the samples having either no odor or a clean, sour odor are marked 0; those with a slightly musty or very slightly putrid odor, 1; those with a musty or slightly putrid odor, 2; those with a distinctly putrid odor, 3.

We are fully aware that no great significance can be attached to numerical expressions of the apparent intensity of a biuret color and still less to that of an odor, and have used these numerals only for convenience of comparison and summation.

It is also recognized that the conditions of our experiments differ from those of commercial pasteurization. On the one hand the temperature at which and time during which our samples were allowed to stand after pasteurization may be somewhat excessive, although the ordinary dealer tends to be less careful of pasteurized than of raw milk. In so far as our conditions are unusual in this respect they would tend to give a more pronounced result, but there is no reason to suppose that they would change it otherwise. On the other hand our conditions are more favorable than those of commercial pasteurization in that any

subsequent contamination is more effectively precluded. The work here reported should be only preliminary to a much more extended study including both chemical and bacteriological observations on a larger number of samples. Lacking the time necessary to make such a study we record the results obtained with the inferences which they suggest in the hope that others may continue the investigation.

The results obtained from these experiments are tabulated below.

While the individual samples show wide variations the general bearing of the results may be summarized as follows:

Pasteurization at 60° appears to have restrained peptonization to about the same extent that it restrained souring. It apparently had no constant effect in rendering the milk either more or less liable than raw milk to the development of offensive odors.

Pasteurization at the higher temperatures (75° and 90°) delayed souring to a much greater extent and had less restraining effect upon peptonization. The samples pasteurized at 75° and 90° developed much more offensive odors than those of the same origin pasteurized at 60° or not at all.

While the development of an offensive putrid odor does not necessarily run parallel either with the ammonia content or with the intensity of the biuret reaction, and while it is evidently incapable of exact measurement it must be regarded as of considerable sanitary significance, since it indicates the predominance of an objectionable type of change.

The results of this investigation together with that of the effect of pasteurization upon the development of ammonia in milk tend to emphasize from the standpoint of the subsequent chemical changes the desirability of low temperatures as recommended by Rosenau and others, in pasteurizing milk when necessary as a safeguard against infectious diseases, and the objectionableness of depending upon pasteurization as a preservative measure.

The importance of keeping milk cold and consuming it quickly are apparently not diminished by its pasteurization even under conditions so favorable as to preclude subsequent contamination.

Group.	Sample No.	ACIDITY.				INTENSITY OF BURET REACTION.				OFFENSIVENESS OF ODOR.			
		Raw.	Pasteurized—			Raw.	Pasteurized—			Raw.	Pasteurized—		
			at 60°.	at 75°.	at 90°.		at 60°.	at 75°.	at 90°.		at 60°.	at 75°.	at 90°.
2 days old.....	1	9.4	2.4	1.3	1.7	1	0	2	2	0	0	2	3
	2	8.3	2.5	1.8	1.7	2	1	2	2	0	2	1	1
	3	7.3	2.6	1.3	1.4	2	1	2	1	0	1	1	1
	4	6.9	1.2	1.5	1.3	2	2	2	0	0	1	1	1
	5	8.1	7.3	1.5	1.3	2	1	1	1	0	0	2	2
	6	7.9	7.5	1.7	1.7	3	2	2	2	0	0	0	0
	7	2.6	1.1	1.3	1.3	0	0	0	0	2	0	2	1
	8	5.6	1.7	1.5	1.5	1	1	1	1	0	0	0	1
	9	8.1	6.3	1.7	1.8	2	2	2	2	0	0	0	1
	Sum	64.2	32.6	13.6	13.7	15	10	14	11	2	4	9	11
4 days old.....	1	8.4	7.3	3.0	3.3	1	1	2	2	0	0	2	3
	2	9.2	8.6	5.7	4.6	1	2	2	2	0	0	2	1
	3	7.5	6.4	4.8	2.2	2	1	2	2	0	0	1	2
	4	8.1	7.6	1.9	1.5	2	2	3	2	0	1	0	1
	5	7.0	7.0	5.1	1.7	2	1	2	2	2	0	2	2
	7	8.8	1.6	1.2	1.2	2	2	2	0	1	1	1	1
	8	7.4	5.4	1.6	1.3	2	1	2	0	0	0	1	0
	Sum	56.4	43.9	23.3	15.8	12	10	15	10	3	2	9	10
Total.....		120.6	76.5	36.9	29.5	27	20	29	21	5	6	18	21



# A PRELIMINARY STUDY OF THE SENSITIZING PORTION OF EGG-WHITE.

By MARY F. LEACH.

(*Western College, Oxford, Ohio.*)

(Received for publication, July 23, 1908.)

This work was undertaken to supplement some work recently published upon the corresponding portion of the cell substance of the colon bacillus,<sup>1</sup> and upon the effects of egg-white and its split products on animals.<sup>2</sup> For some years past researches have been in progress in the Hygienic Laboratory of the University of Michigan upon the bacterial cell, its chemical and physiological nature.<sup>3</sup>

It has been found that by prolonged treatment of the bacterial cell substance with an alcoholic solution of caustic soda, a virulent poison is dissolved out by the alcohol, while in the case of the colon and typhoid bacillus, the part insoluble in alcohol confers immunity to its homologous bacterium. Subjecting egg-white to the same process, it is similarly separated into a soluble portion, which though harmless administered *per os*, is promptly fatal when injected intraperitoneally or intravenously, and an insoluble portion which is not poisonous. It is certainly interesting that a given protein taken into the circulation by way of the ordinary processes of digestion serves to build up the body of the animal and to enable it to carry on its vital processes; while that same protein introduced directly into the circulation can not be thus utilized. A single injection of egg-white has no apparent effect, a second dose within a few days is also well borne, but a second dose after an interval of ten or more days is promptly fatal; thus showing that the first dose has produced sensitization. Now

<sup>1</sup> Leach: On the Chemistry of *Bacillus Coli Communis*, *This Journal*, iii, p. 443, 1907.

<sup>2</sup> Vaughan and Wheeler: *Journ. of Infect. Diseases*, iv, p. 476, 1907.

<sup>3</sup> For bibliography, see preceding reference.

the non-poisonous portion of egg-white sensitizes an animal to egg-white, but not to itself. Thus bacterial cell substance may be separated into poison and immunizing portion, and egg-white by the same method yields poison and sensitizing portion.

*Material.* The whites of fresh eggs were poured into 96 per cent alcohol, the precipitate repeatedly extracted with alcohol, dried upon filter paper, pulverized, thoroughly extracted with ether, again dried and pulverized. After standing in the laboratory for some months, the egg-white thus prepared was boiled with absolute alcohol containing sodium hydroxide according to the method used for bacterial and other proteins.<sup>1</sup> This treatment was repeated three times, experience with other proteins having shown that this is necessary to remove all the poison. The insoluble portion was extracted with alcohol in Soxhlets for three and a half days to thoroughly wash it free from poison and other alcohol-soluble substances. After drying and pulverizing, it is a cream colored powder very similar in appearance to the egg-white from which it was obtained. The poisonous portion, obtained from the alcoholic solution as described in the preceding reference, is a dark brown solid, difficult to dry sufficiently to pulverize it. Both poisonous and non-poisonous fractions give the various protein color reactions, but show striking chemical differences without further purification. The poisonous portion gives a very pronounced Millon test, gives no evidence of carbohydrate, and contains only traces of phosphorus. The non-poisonous fraction gives only a faint Millon reaction, but responds to carbohydrate tests, and contains a considerable amount of phosphorus. These same distinctions have been found in splitting up other proteins by this method.

This preparation of egg-white is markedly hygroscopic, and the split products are still more so. However two to four weeks' drying in an oven not above 103° sufficed to bring them to constant weight. Two samples were cooled in the same desiccator, weighed, returned to the desiccator and weighed again in the same order. Both had gained more at the second weighing than they had lost since the preceding day. On a rainy day sam-

<sup>1</sup> Wheeler: The Extraction of the Intracellular Toxin of the Colon Bacillus, *Journ. of the Amer. Med. Assoc.*, xliv, p. 1271, 1905.



ples would absorb more water during the process of weighing than they had lost in the previous twenty-four hours.

*Ash, nitrogen, phosphorus, sulphur.* For purposes of comparison, ash, nitrogen, phosphorus and sulphur were determined in this preparation of egg-white and the two products obtained therefrom. All determinations were made in duplicate, and the figures given are the averages of closely agreeing determinations. Ash was determined by incinerating to low redness in a platinum crucible, taking pains to avoid volatilizing inorganic matter. Since the phosphorus is originally in organic combination, and is present in the ash as orthophosphate, an amount of  $\text{PO}_4$  is deducted corresponding to the amount of phosphorus present, giving results tabulated as inorganic ash in the second column of the following table, and these results were used in calculating percentages "ash free." Nitrogen was determined by the Kjeldahl-Gunning method, and phosphorus by the Neumann method with modifications.<sup>1</sup> Sulphur determinations were made by fusion with sodium hydroxide and nitrate, and precipitation by addition of hot barium chloride drop by drop. Of course the figures obtained do not correspond with analyses of purified products, such as ovalbumin, ovimucoid, or even of untreated egg-white; but they are of more value in tracing the physiological units than if subjected to further treatment. However the results for egg-white show nitrogen content not far removed from figures for other preparations, but the sulphur is somewhat higher than has been found even in ovimucoid (2.2 per cent).<sup>2</sup> Still it is not unreasonable to suppose that the long continued action of alcohol and ether would give rise to changes aside from those incident to the process of denaturalization. While many observers seem to have ignored the phosphorus in egg-white, Osborne and Campbell<sup>3</sup> found in their four purest preparations of crystallized ovalbumin amounts of phosphorus varying from 0.126 per cent to 0.131 per cent. Certain data in regard to the colon bacillus are appended to the table.

<sup>1</sup> For further details, see paper previously cited On the Chemistry of *Bacillus Coli Communis*.

<sup>2</sup> Mörner: *Zeitschr. f. physiol. Chem.*, xviii, p. 525, 1893.

<sup>3</sup> Protein Constituents of Egg-white, *Journ. of the Amer. Chem. Soc.*, xxii, p. 422, 1900.

TABLE I. PERCENTAGES OF ASH, NITROGEN, PHOSPHORUS AND SULFUR.

	Ash.	Inorganic ash.	N.	P.	S.	N ash free.	P ash free.	S ash free.
Egg-white.....	2.48	2.066	14.48	0.135	2.66	14.7	0.138	2.73
Poisonous fraction.....	1.14		13.74	traces	2.19	13.9		2.22
Non-poisonous fraction..	13.57	12.8	12.67	0.253	2.79	14.53	0.29	3.2
<i>Bacillus coli communis</i>								
Cell substance.....	8.61		10.65	2.87				
Poisonous fraction.....	2.33		11.15	traces				
Non-poisonous fraction	33.25	26.08	5.56	2.34		7.52	3.99	

## SENSITIZING PORTION.

*Comparison with the corresponding fraction of some other proteins.* Edestin, casein, egg-white and the cell substance of *Bacillus coli communis* were all subjected to the action of alkali and alcohol as previously described. The insoluble portion of each, containing the sensitizing or immunizing elements, in all cases gave the various protein color tests, Millon's reaction less markedly than the others, perhaps because of the alkalinity of the preparation. On stirring with water, the edestin preparation was entirely soluble, there was a slight flocculence with the casein preparation, the other two were mainly but not entirely dissolved, but the physiologically active portion of all goes into solution. Addition of a little sodium hydroxide increases the solubility. Mineral acids give a precipitate with the casein and egg preparations.

The most marked difference was found in testing for carbohydrates. As edestin contains no carbohydrate, its preparations of course show no evidence of such groups; however the tests were applied as controls, always with negative results. Although casein is said to contain no carbohydrate, it has been found to respond to the Molisch test<sup>1</sup> and our preparation from casein does also. Reactions previously reported point to the presence of pentose in the bacterial cell preparation.<sup>2</sup> As was to be expected

<sup>1</sup> Mann: *Chemistry of the Proteids*, p. 403.

<sup>2</sup> This *Journal*, iii, p. 453, 1907.

the egg preparation gave evidence of hexose and not pentose. The lead sulfide reaction showed the presence of loosely combined sulfur in the preparations from egg and from edestin, not in the ones from casein and the colon germ.

*Action of acid.* As shown in the preceding paragraphs, qualitative tests indicate the presence of protein, nucleo-compounds and carbohydrates. Mineral acids give a precipitate with aqueous solutions. The method of preparation precludes the presence of free organic acid, but suggests the possible presence of sodium salts of such acids. Accordingly 10 grams of the sensitizing portion of egg-white were stirred with 200 cc. of water and filtered. The filtration was slow, and accompanied by formation of much foam, but finally a clear golden brown solution was obtained. A sample of this solution was titrated with  $\frac{N}{10}$  sulfuric acid, using dimethyl-amido-azobenzol as indicator, until red coloration indicated the presence of free mineral acid. The calculated amount of sulfuric acid was then added to the original solution. On standing the precipitate settled as a tough leathery mass, readily separated from the supernatant liquid by decantation. The precipitate was washed and dried upon a porous plate. It was insoluble in both alcohol and ether, and hence is not fatty acid. It may be added that without further purification it gives the protein color tests, but awaits additional investigation.

*Carbohydrate.* Differences between the poisonous and non-poisonous fractions of a given protein, differences between the non-poisonous fractions of different proteins, and work upon an immunizing product obtained from the non-poisonous fraction of the colon germ, all suggest an investigation of the carbohydrates present. Accordingly samples of the sensitizing portion of egg-white were stirred with water, filtered, and attempts made to separate protein and carbohydrate in the filtrate by means of uranium acetate. The acetate was added both with and without sufficient alkali to keep the solution alkaline. A copious precipitate resulted in both cases, which was filtered out with some difficulty. The slight excess of uranium was removed from the filtrate by addition of sodium phosphate. The filtrate gave evidence of carbohydrate, but the separation was not sufficiently sharp, and the physiological action was lessened, hence that method was abandoned. Acidifying until there was a slight per-

manent precipitate, the addition of either methyl or ethyl alcohol cleared the solution. Phosphotungstic acid precipitated both protein and carbohydrate. In short no method was found that would remove the protein from the solution, leaving the carbohydrate. It is perhaps a legitimate inference that the two are united.

Samples were subjected to hydrolysis and titrated with Fehling's solution. The protein and other compounds present interfered with the reaction, but by adding the solution all or nearly all at once it was possible to obtain comparable results. Experiments with split products from the colon germ gave a maximum reduction by boiling  $2\frac{1}{2}$  hours with 2.5 per cent hydrochloric acid.

Three grams of the non-poisonous fraction of egg-white were mixed with 200 cc. of water and 20 cc. of 25 per cent hydrochloric acid. A second sample was prepared in the same way except that it was filtered before adding the acid. Both were boiled with reflux condenser. After boiling half an hour, and then at intervals of three hours, aliquot parts were removed, neutralized, titrated with Fehling's solution, and amount of reducing substance calculated. Other samples were hydrolyzed with sulfuric acid with less satisfactory results. These preliminary experiments indicated that the reducing substance is all present in the portion soluble in water, and that the maximum yield, which if calculated as dextrose is about 9 per cent, is obtained by boiling 10 to 12 hours until the mixture no longer gives the biuret test.

Accordingly 25 gram samples of the non-poisonous portion of egg-white were shaken for two hours in a mechanical shaker with ten times their weight of water, filtered, 200 cc. more water added, the solution neutralized with hydrochloric acid, then 50 cc. of 25 per cent hydrochloric acid added, thus making approximately a 5 per cent solution of material in 2.5 per cent acid. This was boiled with reflux condenser for 10 to 12 hours, until the solution no longer gave the biuret reaction. It was then filtered, leaving very little upon the filter. The clear red-brown filtrate was cooled, neutralized with sodium hydroxide, and benzoylated by the Schotten-Baumann method. The mixture became very warm, but was cooled by surrounding the flask with pounded ice and salt. When the reaction ceased the compound settled nicely and was filtered with suction after standing two or three hours.

The precipitate was washed with water containing a little ammonia, and treated with boiling alcohol, in which a large portion was freely soluble. On cooling and concentrating the alcoholic solution, a fine yield of crystals was obtained. The crystals from several samples were united and recrystallized from hot absolute alcohol until the solution was water clear and colorless. Macroscopic bundles of needles were thus obtained showing very characteristic grouping. They were washed in alcohol and in ether, dried upon porous plate, the operations repeated until samples from two recrystallizations melted side by side within one or one and a half degrees. The crystals are pure white, readily soluble in benzol, in chloroform and in glacial acetic acid, as well as in alcohol, and melt at  $203^{\circ}$ . When boiled with sodium hydroxide, ammonia is given off; after removing benzoic acid by boiling with hydrochloric acid, the resulting solution reduces Fehling's solution.

0.4150 gm. gave 0.00891 gm. N, corresponding to 2.147 per cent N.

0.4220 gm. " 0.00962 " " " " 2.279 " "

Average = 2.213 per cent N.

These characteristics suffice to identify the compound as glucosamin benzoate which Pumm<sup>1</sup> reports as melting at  $203^{\circ}$ . Kueny<sup>2</sup> prepared different benzoates from glucosamin by varying the conditions of the experiment. The one most readily formed was tetrabenzoate, melting at  $199^{\circ}$  when recrystallized from alcohol, and at  $207^{\circ}$  when recrystallized from glacial acetic acid. He tried by various methods to prepare a pentabenzoate, but without success. Langstein<sup>3</sup> prepared glucosamin benzoate from egg-white, which after once recrystallizing from hot alcohol melted at  $201^{\circ}$  to  $202^{\circ}$ , and gave 1.95 per cent of nitrogen. The theoretical amount of nitrogen in the tetrabenzoate is 2.35 per cent. Thus this benzoate prepared from the sensitizing fraction of egg-white agrees with glucosamin benzoate prepared from glucosamin and from egg-white at least as well as those preparations do with each other. Numerous observers have found glucos-

<sup>1</sup> *Monatsh. j. Chem.*, xii, p. 435.

<sup>2</sup> *Zeitsch. f. physiol. Chem.*, xiv, p. 355, 1890.

<sup>3</sup> Ueber die gerinnbaren Stoffe des Eiklars, *Beitr. z. chem. Physiol. u. Pathol.*, i, p. 83, 1902.

amin in egg-white, and this work shows that it has remained in the sensitizing portion. It is hoped later to investigate the carbohydrate in the corresponding fraction of the colon bacillus.

#### SUMMARY.

Recent articles have described a method for separating bacterial cell substance into a poisonous fraction and an immunizing fraction. Applying the same method to egg-white, it is separated into a poisonous fraction and a sensitizing fraction. The sensitizing fraction of egg-white contains about the same per cent of nitrogen as does the egg-white, and a little higher percentage of sulfur. Evidence of protein, nucleo-compounds and carbohydrate were found; mineral acid gives a precipitate with aqueous solutions, but there was no evidence of fatty acid. Phosphorus, frequently overlooked in analyses of egg-white, remains in the sensitizing fraction, while only traces are found in the poisonous fraction. The carbohydrate and nucleo-compounds of the egg-white remain in the sensitizing fraction, the poisonous portion giving no evidence of such groups. Carbohydrates and protein are apparently united, as different methods of separation fail. After long hydrolysis glucosamin was separated as benzoate. As glucosamin is present in egg-white, it may be inferred that the carbohydrate passes unchanged from the egg into the sensitizing portion.

The experimental work herein described was carried on in the Hygienic Laboratory of the University of Michigan, and I wish to express my indebtedness to Dr. V. C. Vaughan for turning over this problem to me, and for his kindly interest and helpful suggestions during the progress of the work.

## MILK PROTEINS.

By GEORGE A. OLSON.

(Contribution from the Agricultural Chemical Laboratory, University of Wisconsin.)

(Received for publication, July 14, 1908.)

Our present knowledge concerning the protein components of milk is limited to casein, lactalbumin,<sup>1</sup> lactoglobulin,<sup>2</sup> and probably mucoid<sup>3</sup> and opalesin.<sup>4</sup> A number of components, such as fibrin,<sup>5</sup> galactozymase,<sup>6</sup> albumose,<sup>7</sup> gélatine,<sup>8</sup> galactin,<sup>9</sup> lecithin<sup>10</sup> and lactoprotein,<sup>11</sup> have likewise been found. Aside from the proteins mentioned above the writer has isolated a new protein from milk which is characteristically different from any of those alluded to.

*Casein.* When skim milk is treated with dilute acetic acid or on saturation with a salt like sodium chloride a coagulum is formed, which when removed is made up largely of casein, calcium phosphate, water and some fat. On repeatedly dissolving this coagulum with dilute alkali, precipitating with dilute acid and washing the precipitate thus formed with alcohol, ether and alcohol, a product free from calcium phosphate and fat is finally obtained which is known as casein. Casein is a white amorphous body, insoluble in alcohol, ether or dilute acids, but on treatment with alkalies, alkaline carbonates, phosphates or strong acids it

<sup>1</sup> J. Sebelien: *Zeitschr. f. physiol. Chem.*, ix, p. 445, 1885.

<sup>2</sup> J. Sebelien: *Ibid.*: *Journ. of Physiol.*, xii, p. 95, 1891.

<sup>3</sup> V. Storch: *Monatsh. f. Chem.*, xviii, p. 244, 1897; xx, p. 837, 1899.

<sup>4</sup> A. Wroblewski: *Zeitschr. f. physiol. Chem.*, xxvi, p. 308, 1898.

<sup>5</sup> S. M. Babcock: *Soc. for the Prom. of Agric. Sci.*, p. 13, 1898.

<sup>6</sup> A. Béchamp: *Compt. rend. de l'Acad. d. sci.*, lxxvi, p. 836, 1873.

<sup>7</sup> M. R. Palm: *Jahresbericht f. Thierchem.*, xvi, p. 143, 1887.

<sup>8</sup> Morin: *Journ. de phys. et de chim.*, xxv, pp. 428, 431 and 433, 1854.

<sup>9</sup> Selmi: *Gazetta chim. ital.*, iv, p. 482.

<sup>10</sup> Koch: *Zeitschr. f. physiol. Chem.*, pp. 47, 327-330, 1906.

<sup>11</sup> Millon and Comaille: *Jahresber. d. Chem.*, p. 622, 1864.

readily passes into solution. Casein unites with alkaline earths and particularly with calcium phosphate for the association which is present in milk. Rennet splits casein into two proteins one of which (paracasein) in the presence of calcium salts is coagulated forming with the entangled fat of the milk the curd which ultimately becomes the commercial product known as cheese. The other protein, a soluble albumose body (whey albumin) remains in solution. According to Lehmann and Hempel<sup>1</sup> the composition of casein is as follows:

C, 54.0; H, 7.04; N, 15.6; S, 0.771; P, 0.847; O, 21.742.

*Lactoglobulin.* A few milligrams of lactoglobulin per liter of milk can be obtained by saturating the filtrate resulting from the removal of casein by means of sodium chloride with magnesium sulfate. In a moist condition lactoglobulin is neither tough nor elastic and possesses a snow white flaky appearance. Little is known in regard to this protein component of milk.

*Lactalbumin.* On further saturating the lactoglobulin filtrate with ammonium sulfate, Sebelien obtained a protein, lactalbumin, which comprises about one-tenth of the entire protein components of milk. Lactalbumin when dry is a transparent, brittle, hygroscopic mass or a white powder. It is precipitated by hydrochloric acid but dissolves in an excess of the same. Lactalbumin has the following composition:

C, 52.19; H, 7.18; N, 15.77; S, 1.73; O, 22.9.

It differs in composition from casein in that it has no phosphorus, twice as much sulfur, less carbon and more oxygen.

*Opalesin.* Wroblewski has observed an albumin in milk, which is rendered opalescent by the addition of acetic acid. This albumin which he termed opalesin can be salted out with sodium chloride or magnesium sulfate. It is soluble in water and non-coagulable. It gives the color test of albumins, also those not given by casein and is further remarkable for its low carbon and high sulfur content. The composition of opalesin is as follows:

C, 45.0; H, 7.3; N, 15.07; P, 0.8; S, 4.7; O, 27.11.

---

<sup>1</sup> W. Hempel: *Arch. f. d. ges. Physiol.*, lvi, p. 558, 1894.



*Mucoid.* Storch isolated from fat- and salt-free butter serum a protein which after air drying formed a fine loose, hygroscopic powder of grayish white appearance. It is insoluble in water, alcohol, ether and dilute mineral acids. It dissolves in weak and strong sodium hydroxide with difficulty. On treatment with dilute hydrochloric acid this mucoid reduced Fehling's solution. The ash-free mucoid contained 14.2 per cent nitrogen and 2.2 per cent sulfur.

*New protein.* The new protein was first isolated from centrifugal slimes, which were obtained from the inner walls of the bowls of cream separators. Water-free, centrifugal slimes are composed largely of proteins, among which casein is by far the most abundant constituent. The following method was used to isolate the new protein from the slime.

Fresh slime was triturated in the cold with large quantities of 0.5 to 1.0 per cent sodium hydroxide solution. The alkaline extracts thus obtained were filtered and then cautiously treated with 1 per cent hydrochloric acid until precipitation commenced. This precipitate first formed was removed and the filtrate obtained was treated with more hydrochloric acid and the process continued until all of the acid precipitates were removed. When no further precipitation occurred with the acid the process was reversed by adding enough 0.5 per cent sodium hydroxide solution to the acid filtrate until slightly alkaline, removing any precipitate that formed during the process. To every five parts of the slightly alkaline filtrate one part of concentrated hydrochloric acid (sp. gr., 1.20) was added and the entire contents were allowed to stand until the precipitation of the new protein had completely settled to the bottom. This requires from twenty-four to forty-eight hours. The supernatant liquid was then decanted and the remainder filtered. The precipitate was purified by repeatedly dissolving in water and precipitating with concentrated hydrochloric acid, after which it was finally dialyzed, either in parchment tubes or dialyzers, washed in alcohol, ether and finally dried over sulfuric acid. Approximately 1.5 gram of this precipitate was obtained from about five kilos of slime. Sufficient chloroform was used to inhibit bacterial action in the preparation of the protein from slime.

*New protein found in milk, cream and butter.* The presence of

this new protein in whole and skim milk, cream and butter was also confirmed. The casein in the milk and cream was precipitated in the cold by weak acetic acid and removed. The albumin and calcium phosphate were precipitated from the acetic acid filtrate with weak sodium hydroxide solutions and removed. The resulting albumin filtrate was then treated with concentrated hydrochloric acid (sp. gr., 1.20) and allowed to stand over night for the precipitate to settle. Cream appeared to contain a much larger amount than milk. In working with butter, the fat was removed with gasoline and the resulting residue was treated as in the case of the centrifugal slime with positive results.

*Physical and chemical characteristics.* The new protein when desiccated has a brown, varnish-like luster. When dried in the water oven at 95° C. its color does not change. When pulverized it appears as a yellowish white powder. In water it swells and becomes white in appearance. The protein dissolves in weak sodium hydroxide solutions and is precipitated from such solutions by phosphotungstic acid, tannic acid, ferrocyanide of potash, Millon's reagent, concentrated hydrochloric acid, etc., but not with acetic acid. When the protein is added to concentrated sulfuric acid (sp. gr., 1.84) it takes on the white appearance in the cold, but on heating it rapidly chars. It is insoluble in ether or alcohol and gives the biuret reaction. It does not reduce Fehling's solution. Hydrogen peroxide is decomposed by a solution of the protein dissolved in sodium hydroxide solution. Different preparations have been made which before being dialyzed tested from 14, 14.57, 14.28 per cent of nitrogen and after dialyzation tested from 18.43, 18.62, 18.70, 18.81 and 18.93 per cent of nitrogen. The dialyzed protein which contained 18.93 per cent of nitrogen also contained 1.53 per cent of sulfur and 0.811 per cent of ash. The high nitrogen content, together with the other mentioned facts, indicates that this compound is a protein of high complexity. What is more remarkable, the protein when added to sterile milk produces proteolysis.

## MILK ENZYMES.

Among the numerous enzymes which are claimed to be natural constituents of milk may be mentioned galactase,<sup>1</sup> trypsin,<sup>2</sup> amylase,<sup>3</sup> lipase,<sup>4</sup> lactokinase,<sup>5</sup> peroxidase and catalase.<sup>6</sup>

In this paper only proteolytic ferments, such as galactase, trypsin, pepsin or erepsin, are considered, since the specific function (digestion) of any of these has a direct bearing on the work presented in the following pages.

Three distinct methods have been employed in studying proteolysis, namely, the digestive action on milk, the digestive action on milk agar cubes and tests to determine the optimum temperature for its action.

*Proteolytic tests with the new protein.* Twenty series of experiments were conducted with fresh preparations of the new protein in order to learn the nature of the same with reference to proteolysis.

In one series of experiments eleven bottles containing 180 cc. of freshly skimmed milk in each were boiled, cooled, and treated with 5 cc. of chloroform per bottle. Two were kept for control and three lots of three bottles each were treated as follows: In every lot one bottle was made neutral, another bottle, after it had been neutralized, was made acid to the amount of 0.2 per cent of hydrochloric acid and the remaining bottle was made alkaline to the amount of 0.2 per cent of sodium carbonate. In Lots I and III two preparations of the new protein from slime were added. In Lot II a hydrochloric acid precipitate from whey was added. All bottles were made up to equal volumes with sterilized water, and allowed to digest for periods 131 and of 1099 days at room temperature. The bottles were frequently shaken during the earlier part of the digestion period. The total amount of nitrogen in this milk was 0.473 per cent, having a soluble nitrogen content of 0.0283 per cent or equal to 6.026 per cent of the

<sup>1</sup> S. M. Babcock and H. L. Russell: *14th Ann. Rpt. Wis.*, p. 179, 1897, *15th Ann. Rpt. Wis.*, p. 85, 1898, *16th Ann. Rpt. Wis.*, p. 157, 1899.

<sup>2</sup> Moro: *Jahrbuch. f. Kinderheilkunde*, N. F., lvi.

<sup>3</sup> Béchamp: *Compt. rend. de l'Acad. des sci.*, xcvi, pp. 1508-1509.

<sup>4</sup> Marfan and Gillet: *La presse medicale*, pp. 13-16, 1901.

<sup>5</sup> Hougardy: *Bull. acad. roy. belg.*, pp. 888-900, 1906.

<sup>6</sup> Wender: *Oesterr. Chem. Zeit.*, vi, p. 13.

total nitrogen of the milk. All bottles were strong with chloroform at the close of the experiment. The results are as follows:

TABLE I.  
*Proteolytic test of the new protein on milk.*  
*New protein Lot I.*

	131 DAYS DIGESTION.		1099 DAYS DIGESTION.	
	Per cent soluble nitrogen.	Per cent total nitrogen.	Per cent soluble nitrogen.	Per cent total nitrogen.
Control.....	0.0285	6.026	0.062	13.13
Neutral.....	0.0473	9.990	0.340	71.88
HCl, 0.2 per cent .....	0.0550	11.620	0.109	23.04
Na <sub>2</sub> CO <sub>3</sub> , 0.2 per cent.....	0.0547	11.560	0.077	16.28

*Hydrochloric acid precipitate from whey Lot II.*

Control.....	0.0285	6.026	0.062	13.13
Neutral.....	0.0360	7.610	0.058	12.27
HCl, 0.2 per cent .....	0.0295	6.240	0.061	12.89
Na <sub>2</sub> CO <sub>3</sub> , 0.2 per cent.....	0.0495	10.450	0.078	16.39

*New protein Lot III.*

Control.....	0.0285	6.026	0.062	13.13
Neutral.....	0.1275	26.950	0.297	62.79
HCl, 0.2 per cent .....	0.1225	25.900	0.234	49.47
Na <sub>2</sub> CO <sub>3</sub> , 0.2 per cent.....	0.1220	25.790	0.171	36.15

From the data obtained in the above series of experiments it will be noted that at 131 days the largest increase of soluble nitrogen was found in Lot III. While at 1099 days, including the increased soluble nitrogen in the control, it will be observed that the greatest amount of soluble nitrogen was found in the neutral milks and the least in the milks containing sodium carbonate, Lots I and III. It is probable that the increase in soluble nitrogen in the milks made alkaline with sodium carbonate was partly due to the alkali present.

The most important fact developed in this and similar experiments was that the hydrochloric acid precipitates obtained from

whey, which were treated under similar conditions as the precipitates obtained from slimes, did not show any perceptible digestive action, indicating that all external factors that might have been introduced in these preparations and caused proteolysis were removed. Whatever the changes are that took place in Lot II, they are undoubtedly due to some other causes than proteolysis. There is a similarity in the amount of digestion in Lots I and III for 1099 days and in similar experiments where the period was carried on for 1536 days.

*The influence of acid and alkali on proteolytic action.* According to Nasse<sup>1</sup> and Schmiedeberg<sup>2</sup> albumins denaturalize with great rapidity when brought in contact with alkalies and acids.

For this reason it was desired to learn to what extent the acid and alkali to the amount used influenced an increase in the soluble nitrogen and whether or not this power of dissolution was progressive. In this experiment 180 cc. of neutralized and boiled skim milk was added to each bottle. The bottles and contents were then reboiled, cooled and 5 cc. of chloroform was added to each bottle. Two bottles were kept for control, five were made alkaline with sodium carbonate to equal 0.2 per cent solution, and two bottles were made alkaline with sufficient sodium carbonate to make a 0.4 per cent solution. Two bottles were kept neutral and four were made acid with hydrochloric acid to equal a 0.2 per cent solution. A hydrochloric acid precipitate from slime was added to two bottles of milk containing 0.2 per cent of sodium carbonate, two bottles containing 0.2 per cent hydrochloric acid and two bottles of neutral milk. All bottles were made up to equal volumes and then allowed to digest. The total nitrogen in the skim milk was 0.49 per cent with 0.038 per cent soluble nitrogen or equal to 7.75 per cent of the total nitrogen. The results of this experiment are as follows:

<sup>1</sup> O. Nasse: *Arch. f. d. ges. Physiol.*, vii, p. 139, 1872.

<sup>2</sup> O. Schmiedeberg: *Arch. f. exp. Path. u. Pharm.*, xxxix, p. 1, 1897.

TABLE II.

*Influence of acid and alkali on boiled milk.*

	PER CENT SOLUBLE NITROGEN.		PER CENT TOTAL NITROGEN.	
	108 days digestion.	192 days digestion.	108 days digestion.	192 days digestion.
Control.....	0.038		7.75	
HCl, 0.2 per cent .....	0.040		8.16	
HCl, 0.2 per cent .....		0.0435		8.88
Na <sub>2</sub> CO <sub>3</sub> , 0.2 per cent.....	0.0405		8.26	
Na <sub>2</sub> CO <sub>3</sub> , 0.2 per cent.....	0.0410		8.37	
Na <sub>2</sub> CO <sub>3</sub> , 0.4 per cent.....	0.0410		8.37	
Na <sub>2</sub> CO <sub>3</sub> , 0.2 per cent.....		0.0460		9.40

It will be seen from the above data that neither the hydrochloric acid nor sodium carbonate altered the composition of the milk materially. The above results further show that neither the action of hydrochloric acid nor sodium carbonate are progressive.

TABLE III.

*Influence of new protein on same milk as used in Table II.*

	108 DAYS DIGESTION.		1050 DAYS DIGESTION.	
	Per cent soluble nitrogen.	Per cent total nitrogen.	Per cent soluble nitrogen.	Per cent total nitrogen.
Control.....	0.038	7.75	0.078	15.92
Neutral.....	0.124	25.32	0.286	58.37
HCl, 0.2 per cent .....	0.112	22.85	0.150	30.61
Na <sub>2</sub> CO <sub>3</sub> , 0.2 per cent.....	0.118	24.08	0.107	21.84

From the data given in the preceding table it will be seen that the digestive action is similar to that found in Table I, p. 266. Comparing the above data with those found in Table II, p. 268, for the 108 day period it will be seen that the new protein has exercised a pronounced digestive action, while the milk brought in contact with the acid or alkali was practically the same as in the beginning of the experiment as far as the soluble nitrogen is concerned.

*The new protein in relation to its physiological condition.* The protein was heated at different temperatures for periods of ten minutes each. All were cooled to uniform temperature and added to tubes containing milk agar prisms.<sup>1</sup> They were then allowed to stand for three days, observing the condition of the cubes at twenty-four hour periods. In the tubes where digestion took place the action commenced on the outer surface of the cube, dissolving the casein of the milk suspended in the agar inwardly. In a similar manner the filtrate obtained from the separation of a hydrochloric acid precipitate from slime was studied.

TABLE IV.

*Physiological tests with new protein and filtrates from which it was removed.*

	New protein.	Hydrochloric acid filtrate.
Heated at 35° C.....	digestion	
Heated at 40° C.....		digestion
Heated at 45° C.....	digestion	digestion
Heated at 50° C.....		
Heated at 55° C.....		digestion
Heated at 60° C.....	most marked digestion	digestion
Heated at 65° C.....		digestion
Heated at 70° C.....		digestion
Heated at 80° C.....	none.	none.
Heated at 85° C.....		none.
Heated at 100° C.....	none.	none.
Blank.....	none.	none.

It is apparent from the results of the physiological tests given above that the new protein, as well as the filtrate from the same, contains at least one enzyme.

*Proteolytic test with filtrate obtained from new protein.* In the preceding experiment it was learned that the filtrates from the new protein had the function of proteolysis. Owing to the strong hydrochloric acid which was present in the filtrate it was necessary to neutralize the same before adding to a lot of boiled milk which was treated under the same conditions as in previously

<sup>1</sup> E. G. Hastings: 21st Ann. Rpt. Wis., p. 170, 1904.

described experiments. The total nitrogen of the milk was 0.56 per cent, containing 0.063 per cent soluble nitrogen. The results of a thirty day incubation at 39° C. are as follows:

TABLE V.  
*Proteolytic test of hydrochloric acid filtrate.*

	30 DAY DIGESTION AT 39° C.	
	Per cent soluble nitrogen.	Per cent total nitrogen.
Control.....	0.063	11.25
Neutral.....	0.102	18.21
HCl, 0.1 per cent .....	0.1015	18.12
Na <sub>2</sub> CO <sub>3</sub> 0.1 per cent .....	0.1015	18.12

It will be seen from the above data that hydrochloric acid does not completely separate from milk all of the enzyme or enzymes capable of proteolysis.

*Character of decomposition products formed.* It has been shown from the results given in the preceding pages that whenever the new protein was added to milk digestion took place. In the following pages the decomposition products formed by the action of the new protein on milk are compared with those formed by animal ferments and bacteria. The analytical methods followed are the same as those used by Vivian<sup>1</sup> while studying the soluble nitro en compounds formed by galactase.

*Influence of sterilization and chemicals.* In the following table is given the per cents of the various soluble nitrogen compounds present in fresh skim milk, both before and after sterilization, together with the results of the influence of acid and alkali on this milk for periods of 93 and 224 days, respectively. For the study of the acid and alkali influence each bottle containing 200 cc. of sterilized milk plus 5 cc. of chloroform, plus either 10 cc. of  $\frac{N}{5}$  hydrochloric acid and 10 cc. of normal sodium hydroxide, or 10 cc. of water. After corking and sealing, the bottles were placed in the incubator and kept at a temperature of 39.5° C. for the above stated periods with the following results.

<sup>1</sup> A. Vivian: 16th Ann. Rpt. W.S., p. 179, 1899.



TABLE VI.

*Soluble nitrogen compounds in milk as influenced by sterilization and chemicals.*

	Days digestion.	PER CENT OF TOTAL NITROGEN EXPRESSED IN THE FORM OF—				
		Albumose.	Peptones.		Amide.	Ammonia.
			Tannic acid.	Phospho- tungstic.		
Before sterilization.....		2.01	1.24		4.29	
After sterilization.....		1.43	1.23	0.40	4.16	0.00
<i>After sterilization:</i>						
Control.....	93	2.02	1.44	0.42	3.48	0.00
Hydrochloric acid.....	93	1.48	1.88	0.30	4.14	0.60
Sodium hydroxide.....	93	2.78	2.25	0.48	3.23	0.75
Control.....	224	0.50	3.41	0.74	1.45	0.91
Hydrochloric acid.....	224	0.45	2.89	0.72	3.48	1.08
Sodium hydroxide.....	224	1.45	3.30	0.72	3.13	1.08

The most evident feature brought out by this table is the development of ammonia in milk containing acid and alkali at the 93 day and also in the watered milk at the 224 day period. While there was a fluctuation in the albumose content, a decided increase in anti and hemi-peptones was observed in the milk containing acid and alkali. The failure to obtain peptones by phosphotungstic acid before sterilization indicates that a modification had taken place during sterilization.

*Influence of proteolytic bacteria.* According to Effront<sup>1</sup> bacterial spores can retain their secreting power although their ability to germinate has been destroyed. In accordance with this view a series of the same milk under similar conditions as the preceding experiment were inoculated with the *Bacillus subtilis* and allowed to digest for 224 days at 39.5° C. with the following results.

<sup>1</sup> J. Effront: *Monit. Sci.*, 4 Ser., xxi, No. 782, p. 81, 1907.

TABLE VII.

*Influence of Bacillus subtilis in the presence of chloroform.*

	PER CENT OF TOTAL NITROGEN EXPRESSED IN THE FORM OF—				
	Albumose.	Peptones.		Amide.	Ammonia.
		Tannic acid.	Phospho- tungstic.		
Control (amphoteric) .....	0.50	3.41	0.74	1.45	0.91
Control (amphoteric) inoculated.....	0.56	5.98	3.20	13.93	0.90
Hydrochloric acid.....	0.45	2.89	0.72	3.48	1.08
Hydrochloric acid inoculated.....	0.31	5.62	2.71	8.24	0.90
Sodium hydroxide.....	1.45	3.30	0.72	3.13	1.08
Sodium hydroxide inoculated.....	0.72	4.40	1.26	8.53	0.90

In this case it is evident that in spite of the chloroform present, modifications took place in the presence of *Bacillus subtilis*, being most marked in the amphoteric milk. With the exception of the albumose and ammonia contents marked increases in soluble nitrogen compounds were observed in all separations from the inoculated milk.

TABLE VIII.

*Decomposition products formed in the presence of the new protein.*

	PER CENT TOTAL NITROGEN EXPRESSED IN THE FORM OF—				
	Albumose.	Peptones.		Amide.	Ammonia.
		Tannic acid.	Phospho- tungstic.		
Control.....	1.69	4.86	0.85	4.99	0.74
Neutral.....	4.02	14.16	12.05	41.21	0.44
Hydrochloric acid.....	2.75	8.67	2.32	9.18	0.12
Sodium hydroxide.....	2.32	5.49	1.90	6.44	0.13

It will be seen from the above data that the nature of the decomposition products are not due to pepsin digestion, owing to

the comparatively small amounts of albumose present. With the exception of the decomposition products in acid, this digestion agrees fairly well with trypsin which dissociates the albumins into peptones, peptids and finally into amino acids and according to Kutscher,<sup>1</sup> Hirschler,<sup>2</sup> and Stadelmann,<sup>3</sup> into ammonia. It can not be erepsin since this ferment is most active in alkaline solution. The decomposition products closely resemble those formed by bacterial enzymes in the presence of chloroform, which in turn are similar to those obtained with galactase.

*Relation of new protein to galactase and bacterial enzymes.* In the following table the nature of the decomposition products formed from the action of the new protein and *Bacillus subtilis* in the presence of chloroform are compared with Babcock and Russell's results on cheese, galactase, and bacteria and their enzymes in the absence of chloroform, which have been recalculated by the writer on the basis of per cent of total nitrogen. The galactase which these men used in studying the nature of the decomposition products was an extract of fresh centrifugal slime added to sterilized skim milk.

From the data given in the table (p. 274) it will be noted that the decomposition products formed in cheese, by galactase, new protein, *Bacillus subtilis* in the presence of chloroform, and B. 299 at the 28th day are similar. The most marked difference in the decomposition products formed is in the transformations of the more complex albumose and peptones to the formation of less complex end products as amides and ammonia. In regard to galactase digestion the results further indicate that the metabolic processes ceased somewhere between the 28th and the 56th day (4.92 to 6.56 per cent of ammonia) owing to the repressing action of the chloroform present, while on the other hand in the absence of chloroform the metabolic processes were allowed to go on with *Bacillus subtilis*, B. 299, and B. 83, increasing in these cases in ammonia from the 28th to the 112th day. The remarkable, close agreement of B. 299 at the 28th day with galactase at 112th day, and B. 299 with B. 83 at the 112th day, are of note-

<sup>1</sup> F. Kutscher: Marburger Habilitationsschrift, Strassburg, 1899.

<sup>2</sup> A. Hirschler: *Zeitschr. f. physiol. Chem.*, xxxi, p. 165, 1900.

<sup>3</sup> E. Stadelmann: *Zeitschr. f. Biol.*, xxiv, p. 261, 1888.

TABLE IX.  
Comparison of decomposition products.

	Days digestion.	PER CENT OF TOTAL NITROGEN EXPRESSED IN THE FORM OF—				
		Albumose.	Peptones.		Amide.	Ammonia.
			Tannic acid.	Phospho- tungstic.		
Cheese.....	112	4.47	2.52	9.49	17.04	4.75
Galactase.....	28	16.39	9.83	13.11	13.11	4.92
" .....	56	19.67	13.11	9.83	16.40	6.56
" .....	112	9.84	18.03	24.59	16.40	6.56
New protein (a).....	1826	3.93	13.75	11.79	22.59	18.75
New protein (b).....	1096	4.02	14.16	12.05	41.21	0.44
<i>Bacillus subtilis</i> in presence of chloroform.....	224	0.56	5.98	3.20	13.93	0.90
<i>Bacillus subtilis</i> I in absence of chloroform.....	56		3.63	45.45	3.63	27.27
<i>Bacillus subtilis</i> II in ab- sence of chloroform.....	56		5.45	45.45	1.82	20.91
<i>Bacillus subtilis</i> I in absence of chloroform.....	112		3.63	38.88	1.82	37.04
<i>Bacillus subtilis</i> II in ab- sence of chloroform.....	112		10.25	27.47	0.00	39.38
B. 299 in abs. of chloroform.	28	10.00	17.50	10.00	47.50	7.50
" .....	56	0.00	2.25	15.00	60.00	15.00
" .....	112	0.00	0.00	7.25	58.25	28.25
B. 83 in abs. of chloroform.	28	0.00	7.32	12.19	65.85	12.19
" .....	56	0.00	0.00	14.63	53.66	56.13
" .....	112	0.00	0.00	7.35	56.13	36.52

worthy consideration. The new protein (a) contained one spore former per cubic centimeter of milk at the 1826th day. From these deductions it would follow that the decomposition products formed in cheese, by the new protein, *Bacillus subtilis* in the presence of chloroform, galactase, B. 299 at the 28th day, which is nearly quantitatively the same as B. 83 at the 112th day, are collectively speaking the results of bacterial enzymes with the possibilities of more or less metabolic changes.

That galactase is not inherent, but of bacterial origin is contrary to the views held by Babcock and Russell and in conformity

with the views held by earlier investigators, such as Duclaux, Adametz, Weigmann and others who have concluded that bacterial cultures with the aid of their enzymes (casease, Duclaux) can digest casein and form cheese-like odors which are in some cases better flavored than those made under ordinary factory conditions. Adametz and Winkler even went so far as to place upon the market pure cultures under the name of tyrogene<sup>1</sup> for the purpose of ripening cheese.

The conflicting results obtained by Freudenreich and in earlier researches by Babcock and Russell were due to the fact that they believed lactic acid organisms or lactic acid played the important rôle in the ripening of cheese. Chodat and Hoffman-Bang,<sup>2</sup> however, have clearly shown that lactic acid organisms do not digest coagulated casein even in the absence of sugar.

Contrary to the earlier accepted biological views that digestive bacteria are fundamentally important in the process of cheese ripening, Babcock and Russell followed the generally accepted idea that all living cells secrete enzymes and assumed that this phenomenon took place in the milk glands and ascribed the proteolytic changes in milk to this source and named the ferment characteristic in the changes produced in milk and cheese galactase. To support their theory they treated milk with different protoplasmic poisons, such as chloroform, ether, benzol and toluol, assuming that these agents were destructive to bacterial life. In spite of these agents the milk always curdled and finally digestion resulted. It was found, however, that these milks were not sterile "as cultures made in various culture media after the anti-septic had been removed quite often showed bacterial growth." Van Slyke, Harding and Hart<sup>3</sup> in Tables I, II and III of their bulletin in a study on this problem, have clearly demonstrated that the presence of 2.5 to 30 per cent of chloroform had very little influence upon spore formers, there being 25 to 42 germs per cubic centimeter for the first 21 days with a decrease from 6 to 14 germs per cubic centimeter at 192 days in spite of the fact that these samples were shaken daily. They also concluded

<sup>1</sup> *Molkerei Ztg.*, xiv, p. 817, 1900.

<sup>2</sup> *Ann. inst. Pasteur*, xv, p. 36, 1901.

<sup>3</sup> *Bull. No. 203, N. Y. Exp. Sta.*

from their experiments that chloroform had very little influence upon the enzymes present.

Numerous investigations<sup>1</sup> have been conducted in order to test the germicidal effect of chloroform. The results of these investigations clearly show that the vegetative forms of bacteria are destroyed in the presence of chloroform while the spores are able to survive for long periods of time.

Owing to the danger of including bacteria from external sources Babcock and Russell<sup>2</sup> next endeavored to obtain milk under aseptic conditions, and after being placed under antiseptics these milks also showed an increase in the soluble nitrogen content with each succeeding analysis. That these milks were obtained under aseptic conditions does not imply that they were sterile for as they have stated<sup>3</sup> "after being exposed to the action of antiseptics for a time these milks were found to contain bacteria, but as they were spore bearing forms entirely, the presumption is that they existed in the milk in a latent condition." According to Van Slyke, Harding and Hart<sup>4</sup> only one sample of milk was obtained by them which was germ-free and this one was not studied for proteolytic changes. Boekhout and De Vries<sup>5</sup> drew 200 samples of milk containing 15 cc. in each from one cow and 120 samples of milk containing 15 cc. in each from another. It is a noteworthy fact that out of the 320 samples of milk obtained not one of them was germ free. Some of the bacteria present were able to coagulate the milk in a longer or shorter time, while others were able to digest casein. These samples of milk, together with those obtained at the New York Experiment Station were drawn under aseptic conditions. Ward<sup>6</sup> found in nineteen udders examined "that the lactiferous ducts harbor bacteria throughout their whole extent." Granting that sufficient quantities of germ-free milk can be obtained for proteolytic studies,

<sup>1</sup> M. Kirchner: *Zeitschr. f. Hyg.*, viii, pp. 465-489, 1890; A. B. Green: *Centrabl. f. Bakt.*, I Abt., Ref., xxxv, p. 804, 1905; Corini: *Ibid.*, I Abt., Ref., xxxvi, p. 47, 1905; A. H. Nijland: *Arch. Hyg.*, lvi, p. 361, 1906.

<sup>2</sup> *Rpt. Wis. Exp. Sta.*, p. 174, 1897.

<sup>3</sup> *Ibid.*, p. 175, 1897.

<sup>4</sup> *Bull. No. 203 N. Y. Exp. Sta.*

<sup>5</sup> *Centralbl. f. Bakt.*, II Abt., vii, p. 826.

<sup>6</sup> *Bull. No. 178, Cornell Exp. Sta.*

the possibilities of bacterial enzymes will always confront us for (if the writer is not mistaken on this point) no milk has been obtained from the entire quarter of an udder which is absolutely germ free. Further it is reasonable to believe that a bacterial enzyme once formed would be distributed throughout the entire mass.

Many of the bacterial forms present in the udder are of a proteolytic type and in consequence we should expect bacterial growth, resulting in the production of enzymes before the milk was ever drawn. At most dairy farms cows are milked at intervals running from ten to twelve hours apart, leaving ample time for the production of sufficient quantities of bacterial enzymes to bring about the necessary transformations of the casein in milk.

In Babcock and Russell's experiments the milk was placed in 2.5 liter bottles, leaving approximately one-half liter air space in every bottle. The specific gravity of the chloroform (1.4) which was added to the milk, or vice versa, is heavier than milk (sp. gr., 1.032) and nearly all settles to the bottom. Under this condition the upper strata of milk, together with what milk adheres to the walls of the container above the milk, contain very little chloroform, if any, and therefore permit of ocean beds for the bacteria to grow in. Bacterial growth means the formation of more enzymes, and if the bottles are shaken at twenty-four hour intervals an additional amount of enzyme results with each shaking until the quantity of ferment has been increased to an amount that will hasten the curdling of the milk and digest the casein. The by-products formed, together with the chloroform present in the milk, tend to devitalize the spore formers and they finally become extinct. This is undoubtedly what has taken place in the investigations conducted by Babcock and Russell, who went on the assumption that 2 to 3 per cent of chloroform would keep a milk sterile.<sup>1</sup>

In reviewing their work on the antiseptic effect of various substances on milk,<sup>2</sup> we learn that their means of estimating the efficiency of various agents is based upon the agent which is most suitable to allow spore formers to survive and at the same time

<sup>1</sup> *Rpt. Wis. Exp. Sta.*, p. 103, 1898.

<sup>2</sup> *Ibid.*, p. 99, 1898.

be destructive to the lactic acid organisms present, for all milks which soured with certain antiseptics were discarded for as they have said<sup>1</sup> "2 to 3 per cent of chloroform was sufficient to keep the milk from souring."

*Method of isolating galactase.* In several periodicals and text books issued during the past few years the statement has frequently occurred that Babcock and Russell isolated the enzyme galactase. As a matter of fact the enzyme galactase (meaning by this an inherent enzyme) has never been isolated. In 1901 the writer, then a student of Dr. Babcock, studied the protein composition of centrifugal slimes with a special effort to isolate the ferments present therein, which resulted in the finding of the new protein. In the Fourteenth Annual Report, p. 178, 1897, is given the method used by Babcock and Russell for isolating galactase and in short is as follows: Equal quantities of slime and 40 per cent alcohol were worked into a paste and finally the coarser particles of suspended matter were removed. To the resulting extract thymol or benzol was added in order to allay any possible fermentation. This extract, after standing twenty-four hours, was then concentrated to one-tenth its volume at a temperature not exceeding 25° C. and finally neutralized with sodium carbonate and added to milk with the results of very rapid digestion.<sup>2</sup> It is needless to say that every step undertaken in their procedure of isolating galactase, with the single exception of the antiseptic added, was favorable to bacterial growth to say nothing of the number of bacteria plus their enzymes previous to such an undertaking. In the experiments where these concentrated bacterial extracts plus their enzymes were added to milk we have data<sup>3</sup> which are in accord with the results obtained by the writer (see Table IX), with the exception that the writer worked with a specific digestive organism rather than a possible number of digestive organisms.

Referring to Table IX it will be seen that the decomposition products formed by *Bacillus subtilis* and its enzyme in the presence of chloroform are sharply contrasted from those resulting

<sup>1</sup> Rpt. Wis. Exp. Sta., p. 86, 1898.

<sup>2</sup> Ibid., p. 179, 1897.

<sup>3</sup> Ibid., p. 165, 1899.



from the action of the *Bacillus subtilis* and its enzyme in the absence of chloroform, where 76 per cent of the soluble nitrogen was in the form of peptones (by phosphotungstic acid) and ammonia. Owing to the repressive action of chloroform upon the vital processes of the *Bacillus subtilis*, there are practically, under this condition, only specific chemical changes which take place, viz: the digestive action of the bacterial enzyme, while on the other hand in addition to these changes there are also chemical changes taking place in the organic body.

*Importance of enzymes in cheese ripening.* That enzymes play an important rôle in the ripening of cheese is admitted. Duclaux<sup>1</sup> as long ago as 1880 advanced a theory believing that the enzyme casease derived from the Tyrothrix organism (allied to *Bacillus subtilis*) brought about the characteristic changes in the process of cheese ripening. Duclaux, Adametz, Weigmann and their pupils have shown that pure cultures of organisms with the aid of their enzymes can digest casein, sometimes resulting in flavors superior to those found in the usual make of cheese. Freudenreich found in Emmenthaler the lactic acid organism, *Micrococcus casei liquefaciens*. According to Peterson<sup>2</sup> this organism is frequently met with and increases rapidly during the first ten days of curing. Other microorganisms have been found which play an important rôle in the ripening of cheese, viz: *Bacillus casei limburgensis* in Backsteinkäse, *Micrococcus casei liquefaciens* and the *Bacillus casei limburgensis* combined have a powerful action in Camembert cheese,<sup>3</sup> *Penicillium album* in Briekäse, *Penicillium glaucum* in Roquefort and a kopfschimmel in gamelost. Some forms of mold<sup>4</sup> are capable of dissolving a large part of the casein and according to Rahn<sup>5</sup> there is present in the hard type of cheese a yeast besides lactic acid bacteria.

In earlier researches investigators failed to find digestive organisms in the hard type of cheese. It is now known, that digestive bacteria are present in cheddar cheese as well as in other forms and are generally all found in colonies.

<sup>1</sup> *Journ. Amer. Chem. Soc.*, p. 436, 1882.

<sup>2</sup> *Troili Peterson s. Ann.* 3 S., 487.

<sup>3</sup> Epstein: *Arch. f. Hyg.*, xliii, p. 1.

<sup>4</sup> Tiechert: *Milch. Ztg.*, p. 786, 1903.

<sup>5</sup> Rahn: *Centralbl. f. Bakt.*, II, xv, p. 786.

The conformity of results obtained at the Wisconsin and New York Experiment Stations in experiments with normal and chloroformed cheese is in favor of bacterial action. The presence of chloroform in cheese as in the case with milk, represses bacterial action, thus practically preventing chemical changes within the organic body, while the bacterial enzyme is left free to act.

It will be seen from the discussions presented in the preceding pages that the question rests upon the following thought, i.e., whether or not the enzymes present in milk or cheese and carry on the important rôle in the processes of ripening have originated from an inherent source or from bacteria, yeast or molds. The fact that digestion is most marked in milk and cheese in the presence of bacteria, the finding of digestive bacteria in udders, the resistance of spores to chloroform, the ability of *Bacillus subtilis* enzyme to form similar changes in the milk as in the case of galactase, the failure of Salkowski to find demonstrable evidence of digestion in two samples of normal milk in the presence of 0.3 per cent of chloroform (undoubtedly due to the absence of proteolytic enzyme secreting organisms) all indicate that the proteolytic enzyme or enzymes present in milk have originated from one source, namely, bacteria.

Since centrifugal slime, milk, cream, etc., generally contain large numbers of bacteria it may be that the new protein is a resultant of bacterial action. The fact that the new protein digested sterilized milk, as well as the fact that enzymes attach themselves to materials in suspension (as in the case of the formation of the new protein) together with the fact that the bacterial action is certain before, if not during, preparation, thereby forming large quantities of bacterial enzymes, all tend to indicate that the characteristic digestion of the new protein is one of incorporation.

The failure to obtain digestion with precipitates prepared from cheddar cheese whey indicates one of two things, that either the ferment is incorporated with the curd or the amount present in whey was so diluted that what little was present was injured by the action of strong hydrochloric acid.

## CONCLUSIONS.

(1) By the careful removal of the casein and albumin in slime with weak acid and alkali, a filtrate is obtained in which a new protein can be isolated by the addition of one part of concentrated hydrochloric acid (sp. gr., 1.20) to every five parts of the filtrate.

(2) This new protein is found in milk, cream and butter.

(3) Chemically the new protein is very rich in nitrogen, containing 18.93 per cent of the same. It gives the biuret reaction and is dissolved in weak sodium hydroxide solutions.

(4) Physically the new protein possesses a brown varnish-like luster which when pulverized is changed into a whitish appearance. In water it swells and takes on a whitish appearance.

(5) When added to milk a part of the casein is digested in the same. Digestion is most favorable in neutral milks on prolonged standing.

(6) Physiologically the enzymic properties are most active at a temperature of 65° C. At 80° C. the ferment was destroyed.

(7) The filtrate obtained after the removal of the new protein also has digestive properties.

(8) The influence of chemicals and sterilization tend to slightly modify the soluble nitrogen compounds of the milk.

(9) The addition of digestive bacterial cultures to sterilized milk in the presence of chloroform caused proteolysis.

(10) The decomposition products formed in the presence of the new protein are similar to those formed in the presence of galactase and bacterial enzymes under the same conditions. From these facts it is believed that the characteristic digestion of the new protein and galactase are of bacterial origin.

(11) The enzymic property of the new protein is one of incorporation.



# THE USE OF THE FERMENTATION TUBE IN INTESTINAL BACTERIOLOGY.

By C. A. HERTER, M.D., AND A. I. KENDALL, Ph.D.

(Received for publication, July 5, 1908.)

During the progress of a series of investigations bearing upon the bacterial flora of the intestinal tract of infants and adults in health and disease, the writers have obtained valuable information from the routine use of the fermentation tube, by using methods similar to those of Dr. Theobald Smith,<sup>1</sup> who has repeatedly called attention to the fundamental importance of this apparatus in the study of fermentative bacteria. His researches have shown conclusively that the gas volume, gas ratio (proportion of gas soluble in caustic alkali to the insoluble portion) and the length of time necessary to produce the maximum amount of gas are characteristics of prime importance in the study of this group of organisms. Furthermore, by the simple addition of bits of fresh sterile animal tissue<sup>2</sup> he has succeeded in cultivating in these tubes a number of anaërobes which would not grow under ordinary conditions.<sup>3</sup> Finally, by introducing the use of milk in

<sup>1</sup> Das Gährungskölbchen in der Bakteriologie, *Centralbl. f. Bakt.*, vii, pp. 502-506, 1890; Einige Bemerkungen über Säure- und Alkalibildung bei Bakterien, *Ibid.*, viii, p. 389, 1890; The Fermentation Tube, etc., *Wilder Quarter-Century Book*, pp. 187-234, 1893.

<sup>2</sup> *Centralbl. f. Bakt.*, vii, pp. 502-506, 1890.

<sup>3</sup> There seems to be considerable question about the priority of this procedure. According to Marino (*Méthod pour isoler les anaérobies*, *Ann. de l'inst. Pasteur*, xxi, p. 1005, 1907) the credit belongs to Duen-schmann (*Étude expérimentale sur le charbon symptomatique et ses relations avec l'oedème malin*, *Ann. de l'inst. Pasteur*, p. 482, 1895) who mentions the fact that Roux, under whose direction this work was carried out, had previously used serum (beef) to cultivate certain anaërobes. As a matter of fact Theobald Smith (*Das Gährungskölbchen in der Bakteriologie*, *Centralbl. f. Bakt.*, vii, p. 502, 1890) very distinctly mentions the fact that sterile animal tissue may be employed to advantage in the cultivation of certain obligate anaërobes and he actually employed fermentation tubes enriched in this manner at that time.

## 284 Fermentation Tube in Intestinal Bacteriology

the fermentation tube, he has added much to the value of this medium for bacterial research.<sup>1</sup>

Smith's published results have been obtained chiefly with pure cultures. Herter and Ward<sup>2</sup> have studied the gas volume with fermentation tubes inoculated with mixed intestinal flora and Herter<sup>3</sup> has studied the sediments derived from the inoculation of such tubes.

### TECHNIQUE.

(1) *Preparation of sample.* It is necessary at the start to have fresh specimens of stools for investigation—samples that have stood for some time or have been freely exposed to the air or to high temperatures are liable to change rapidly in their bacterial composition. Nonspore-forming anaërobes may rapidly succumb while, coincidentally, hardy forms multiply until they exist in abnormally large numbers, thus influencing very markedly the bacterial aspect of the result.

It is necessary in this particular line of investigation for several reasons to inoculate appropriate amounts of fecal material; the portion added must not be too great or the acids and other antagonistic products formed by the more rapidly growing facultative organisms will seriously inhibit the growth of the more strictly parasitic types; too small an amount, on the other hand, may fail to furnish a representative growth of significant microorganisms. The less numerous but perhaps extremely important forms may, without these precautions, be overlooked and missed entirely.

For routine purposes one gram of feces thoroughly emulsified in ten cubic centimeters of physiological saline solution is an

<sup>1</sup> Milk intended for the fermentation tube must be sterilized at least four successive times at appropriate intervals to insure the absence of resistant spore-forming bacteria which ordinarily escape observation. After sterilization and before inoculation milk fermentation tubes must be incubated several days at body temperature to test their sterility. See Smith, Brown and Walker: *The Fermentation Tube in the Study of Anaërobic Bacteria with Special Reference to Gas Production and the Use of Milk as a Culture Medium*, *Journ. Med. Research*, xiv, pp. 193-206, 1905.

<sup>2</sup> This *Journal*, i, p. 415, 1906.

<sup>3</sup> *The Common Bacterial Infections of the Digestive Tract*, 1907.

excellent dilution. Probably all types present that are capable of development in fecal media are thus represented.<sup>1</sup> If mucus is present in the stool it should be washed in sterile water and inoculated separately. One cubic centimeter of the suspension is placed in each tube.

Certain forms, as for example, the *B. bulgaricus* described by Metchnikoff, and many alkali-producing bacteria, will not grow in the ordinary fermentation media but usually develop rapidly in milk fermentation tubes.

The period of incubation is a very important factor. Experience has shown that during the first eighteen to twenty hours (rarely longer than this) the majority of the vegetative cells will be at their maximum growth; after this time, owing partly to antagonism, partly perhaps to the fact that the nutrient material that was carried over with the suspension is exhausted, many forms die out, while the more saprophytic organisms increase enormously.

The actual gas volume is rather less at twenty hours than at subsequent periods as a rule, but the relative value of this feature is at the maximum. The bacteria derived from ordinary stools, particularly of the colon type, tend to attain a more or less constant gas volume after forty-eight hours.

In plain bouillon without the addition of carbohydrates (particularly with media made from meat juice instead of meat extract as a basis, gas is sometimes liberated after acidification of the culture with hydrochloric acid, although no gas was present during the incubation period. Also the addition of cystin to the medium tends to increase the gas volume. This gas is hydrogen sulphide, and its total amount may be estimated with a considerable degree of accuracy through the absorption effected by the addition of a soluble salt of a heavy metal.

The gas ratio is not an especially important characteristic in mixed fecal flora, much less so in fact than is the case with pure cultures. Frequently the volume is too small to measure, and

<sup>1</sup> The emulsion should be rapidly prepared and inoculated. Too long an exposure in the relatively aerobic saline solution may, and frequently does, eliminate many vegetative and nonspore forming anaerobic forms, particularly those of infant flora, while the more vigorous aerobic bacteria increase rapidly.

## 286 Fermentation Tube in Intestinal Bacteriology

the inability to decide offhand which organisms are concerned in its elaboration makes the determination at best of doubtful value.

Of the greatest importance, on the contrary, is the character of the sediment. Smith<sup>1</sup> has shown that the deposit at the base of the closed arm is an excellent source of material for reinoculation with anaërobic bacteria (using pure cultures) and our observations have demonstrated in addition the fact that certain organisms assume characteristic appearances which in some instances are even diagnostic.

For making suitable smears the material is best removed by a finely drawn out capillary pipette, then spread upon slides, using the tip of the pipette as a spreader. The excess of solution runs back into the pipette by capillarity, leaving a thin, uniform smear which dries quickly and stains readily.

Gram's staining method followed by dilute carbol fuchsin or anilin-oil safranin furnishes the most distinctive preparations.

2. *Fermentative media.* For ordinary purposes, the regular dextrose, lactose and saccharose bouillons are employed. Freshly sterilized media are desirable because the anaërobic condition is much better developed in the closed arm in such media.

For special investigations, where it is necessary to be absolutely certain of the reaction of an organism upon different sugars, one of us (A. I. K.) has succeeded in eliminating the chief sources of error due to heating carbohydrate media, namely, the caramelization and inversion. This is conveniently accomplished by sterilizing the sugar separately (as a 10 per cent solution) by two passages through appropriate Berkefeld filters. The sugar solution is aded to the nutrient bouillon (freed from fermentable substances by Smith's method) in such amounts that a 1 per cent solution of fermentable carbohydrate results.<sup>2</sup> The bouillon is

<sup>1</sup> Das Gährungskölbchen in der Bakteriologie, *Centralbl. f. Bakt.*, vii, pp. 502-506, 1890.

<sup>2</sup> Liborius (*Zeitschr. f. Hyg.*, i, p. 116, 165, 1886) was the first to recognize the importance of sugars and particularly dextrose, as a nutritive and reductive medium. He used 2 per cent, and his example has been followed without question by the majority of English, French and German investigators. Smith (*Centralbl. f. Bakt.*, xviii, p. 7, 1895; xxii, p. 49, 1897) also finds dextrose very important for the development of anaërobes but his



sterilized in fermentation tubes in the usual manner, adding rather less than usual to provide space for the carbohydrate which is introduced later.

Media made up in this way have one disadvantage, namely, the lack of absolute anaërobiosis that obtains when they are sterilized as one solution. This objection is more imaginary than real, however, because experience has shown that intestinal organisms capable of growing in pure culture in the ordinary single solution media will grow quite as well in the double solution medium.

#### RESULTS.

Herter and Ward<sup>1</sup> found, using dextrose, Schering's diabetin (dextrose-lævulose mixture), lactose and saccharose, the following average amounts of gas produced by normal stools (sixteen in all):

Dextrose.	Dextrose-lævulose.	Lactose.	Saccharose.
26.75	27.50	29.9	19.5 mm.

These fermentation tubes had anaërobic arms approximately 95 mm. long. As a rule the lactose tube showed the most gas; saccharose the least. In conditions of disease the gas volume varied greatly—in certain instances 50 per cent of the vertical limb was filled; in other cases no gas was found.

Our own series indicate in addition that feces derived from many individuals harboring *Bact. Welchii* (the gas bacillus) may form extremely large volumes of gas—even 90 to 100 per cent of the whole tube—although the average is much less, usually about 45 mm. Pure cultures of *B. coli* form about 30 mm. of gas under similar conditions.

Coccal forms when present in numbers, generally inhibit gas formation: gas may even not be formed at all in certain cases, although gas-forming organisms are present. Normal infants, particularly those that are exclusively breast-fed, form as a rule rather less gas than adults. The volume varies with age and con-

results show that 2 per cent is too great a quantity and that 1 per cent is far preferable. Marino (*loc. cit.*) finds from 0.3 to 0.5 per cent even better and our experience indicates that the lesser amounts are preferable in many instances.

<sup>1</sup> *Loc. cit.*

## 288 Fermentation Tube in Intestinal Bacteriology

dition but fifteen to twenty millimeters represents fairly the average. The amount furthermore depends upon the relative proportion of *B. bifidus* present; if the latter organism be abundant, less gas is formed, because this species produces sufficient acid to inhibit the growth of the ordinary gas-formers.

Diarrhoeal stools vary in their gas production. In those cases where large numbers of cocci are brought down from higher levels of the intestine, relatively small gas volumes are the rule, while in similar movements associated with large numbers of colon bacilli, or with organisms of the *lactis aërogenes* type, a much greater production takes place.

Mention has already been made of the fact that the fermentation tube is a particularly simple and efficient apparatus for cultivating anaërobic intestinal bacteria. Of these organisms a few will not grow in pure culture under the same conditions, although they usually thrive symbiotically with facultative anaërobes.

There can be no doubt that certain substances, particularly favorable for the growth of these more or less strictly parasitic forms are carried into the fermentation tube as a part of the fecal suspension, and during the first eighteen or twenty hours furnish a suitable pabulum for their growth—material, furthermore, which is not present in the fermentation tube as it is ordinarily made up. It is extremely probable that their growth is further aided by the presence of more readily growing bacteria which frequently render the tubes extremely anaërobic by the removal of the last traces of dissolved oxygen.

In the fermentation tube every transition from almost complete anaërobiosis to aërobiosis obtains and it is possible for bacteria to find almost any tension of oxygen from more or less complete saturation in the bulb to practically its entire absence in the closed arm.

With such favorable conditions—proper food supply and gaseous environment—the growths are very varied and in a measure representative of the organisms originally present in the feces. This fact is best appreciated after one examines the sediments, particularly those stained by Gram's method followed by the counter stain mentioned above.

The organisms are as a rule much more characteristic morphologically in the deposit at the bottom of the closed arm than is the

case in the feces from which they were derived, because the majority are in what may be termed the "active" vegetative stage. Bacteria in this condition are larger and more nearly typical than under conditions where they have become attenuated and degenerate in their morphology, as frequently happens in the case of constipated stools. The staining reactions also are much sharper and more distinctive at this period.

Perhaps the most striking example of the differentiation one may ordinarily meet with in a sediment from a fermentation tube is that shown by a common bacterium in infants' stools called by Tissier, its discoverer, *B. bifidus communis*. This organism is an anaërobic Gram-positive bacillus, frequently occurring with rather pointed ends in normal infants' stools; not readily recognized and not especially characteristic. Furthermore it is not an easy organism to cultivate in ordinary media. In fermentation tubes, however, it grows rapidly and at the end of eighteen hours shows the peculiarly striking bifid ends to which it owes its name. This fact, judging from the literature published upon the subject so far, has hitherto been unrecognized, but it appears to be characteristic, of great constancy, and a unique example of the value of such examinations. This organism will not grow, or at least only slightly, in fermentation media in pure culture. If, however, one adds a bit of sterile animal tissue, or inoculates directly from a stool, together with other bacteria, the growth is marked.

It is advisable, and frequently necessary, to use fermentation tubes containing plain bouillon instead of the regular fermentation media. Certain bacteria will not grow well where fermentable sugars are present, while others are rapidly eliminated as the medium becomes acid. *B. putrificus* is a good example of such an organism. Sediments derived from plain bouillon in fermentation tubes, particularly those which are rendered more suitable by the addition of sterile animal tissue, frequently show anaërobic growths that would not be included in carbohydrate solutions.

One point in connection with the fermentation tubes deserves special mention—gas volumes are frequently variable with the same individual and it is necessary to cover considerable periods of time before assigning a special value to this factor in individual cases or attaching much importance to deviations from the average.

## 290 Fermentation Tube in Intestinal Bacteriology

Among the organisms ordinarily met with in the feces, a few of the more important may be mentioned:

(1) *Bact. Welchii*, a thick, rather large, strongly Gram-positive bacillus. In large numbers they give rise to a considerable augmentation of the normal gas volume, so that the amount is frequently twice the normal.

An organism described by Herter<sup>1</sup> resembles the gas bacillus morphologically but does not form gas.

(2) *B. coli*. Short bacilli, about one micron in diameter, Gram-negative. These organisms usually determine the gas volume and it is chiefly to their action that the normal gas volume is due.

(3) *B. lactis aërogenes*, somewhat more oval than the colon bacillus and like that organism, Gram-negative. This form is not particularly common in the stools of adults<sup>2</sup> but is present usually in the excreta of bottle-fed infants and tends to increase the gas volume, if numerous.

(4) Coccal forms, usually Gram-positive. These bacteria produce as a rule considerable amounts of acid but no gas, and inhibit to a considerable degree the fermentative action of the above mentioned forms.

(5) A Gram-positive bacillus with bifid ends (*B. bifidus*). It is very common in the stools of breast-fed infants. When this organism is present in numbers, the amount of gas is usually considerably reduced. Its inhibitory action is due, as is the case with the coccal forms, to its excessive acid production.

In several instances, *B. bifidus* has been isolated from mucus, while the remainder of the stool was almost devoid of these forms.

The full significance of the fermentation-tube sediments cannot be regarded as completely worked out. It is a striking peculiarity of the growths in the sediments that they frequently do not show a multiplication of microorganisms closely representative of the varieties which are seen in the Gram-stained fields of the feces. This failure in correspondence between the characters of the dominant organisms in the fermentation tubes on the one

<sup>1</sup> *Loc. cit.*

<sup>2</sup> MacConkey (Lactose-fermenting Bacteria in Feces, *Journ. of Hygiene*, 1905, pp. 333-379) found it in 4 out of 625 lactose-fermenting cultures from normal stools, both animal and human.

hand and the feces themselves on the other, depends largely upon the fact that the nutrient conditions are ordinarily radically altered by the transfer to the fermentation media. This alteration in medium makes it possible for types of bacteria not obviously dominant in the feces, or, indeed, clearly in the minority, to gain a relatively prominent position under these conditions.

The fact that such a readjustment of types is liable to occur has important advantages and equally significant drawbacks. Without recognizing the disproportionate growth one might erroneously assume that a much larger portion of a certain flora is present than is the case; for example fecal fields may contain small numbers of *B. bifidus*, yet in the fermentation tubes they may be prominent. Again, the gas bacilli may undergo extensive multiplication in the fermentation tube despite the fact that the fecal material from which they were obtained contains them in moderate numbers only. Here again one sees the necessity for controlling the appearances obtained from the sediments of the fermentation tubes by means of cultures from the stools as well as by close examination of the Gram-stained fecal fields. Similar overgrowths occur with the coccal forms, *Mic. ovalis* (*enterocoque*), streptococci and staphylococci.

The disproportionate growth has its advantages as well as its disadvantages. Certain types which are significant although originally occurring in small numbers are thus brought to notice when otherwise they would be overlooked.

Experience has shown that overgrowths occurring during the first eighteen to twenty hours of incubation are due to the presence of significant numbers in the stools, capable of asserting themselves in the higher levels of the digestive tract, and capable of enormous proliferation under suitable nutrient conditions. An excellent example is again furnished by *Bact. Welchii*. In patients who have an infection of the intestinal tract with this organism there may be times of improvement when the numbers of this particular type in the feces is small, as shown by the microscopic examination of the fecal fields—so small, in fact, that if the observation were confined to the patient at this time, no suspicion would be excited of the existing tendency of overgrowth of these organisms in the intestine. Yet upon inoculation of the feces into fermentation tubes a prominent, active

## 292 Fermentation Tube in Intestinal Bacteriology

growth of these organisms is very liable to occur under these conditions.

In contrast with this is the following observation. The fecal fields from normal nurslings and bottle-fed children commonly show a few organisms having the morphology of the gas bacillus. That these bacteria belong in the class of the gas bacilli is made probable through the fact that by inoculating relatively large amounts of the feces into rabbits' ear-veins, with subsequent incubation (Welch-Nuttall test) the typical gas-liver will be developed. Inoculations into fermentation tubes made from feces of this type of case have, in our experience, failed uniformly to show overgrowths of this bacillus.

It should be clearly understood that the presence of moderate or even considerable numbers of *Bact. Welchii* does not necessarily lead to overgrowth in the fermentation tube.

# AN OBSERVATION ON THE FATE OF *B. BULGARICUS* (IN *BACILLAC*) IN THE DIGESTIVE TRACT OF A MONKEY.

(Plates I-III).

BY

C. A. HERTER

AND

A. I. KENDALL.

(*Fellow of the Rockefeller Institute.*)

(Received for publication, August 12, 1908.)

The incentive to make the observations here recorded came from certain problems suggested by the rapidly extending use of artificially soured milk which is following closely upon the publication of Metchnikoff's highly speculative volume, entitled "The Prolongation of Life." Really decisive experiments bearing upon the value of what may be termed "sour milk prophylaxis" are not numerous and even Scriptural quotations have been invoked to lend color to, and enhance the meager supply of literature. One of us<sup>1</sup> has studied the effect of introducing large numbers of *B. coli*, *B. proteus vulgaris* and *B. acidi lactici* into the intestinal tract of dogs to determine their action upon indol production. Living cultures of *B. coli* and *B. proteus*, for example, caused an increase of indican and of ethereal sulphates in the urine.<sup>2</sup> Killed cultures prepared in the same manner gave little or no increase in these putrefactive constituents.

<sup>1</sup> C. A. Herter: On Certain Relations Between Bacterial Activity in the Intestinal Tract and the Indican of the Urine. *Brit. Med. Jour.*, 1897, ii, p. 1847.

<sup>2</sup> Precautions were taken to prevent the introduction of putrefactive products derived from the media upon which the bacteria were grown. The organisms were cultivated upon slanted agar (removed by careful washing with salt solution, avoiding the inclusion of any portion of the agar) and then injected.

Lactic acid bacteria injected directly into the small intestine in similarly conducted experiments showed a tendency to cause a reduction in the output of indican and ethereal sulphates.

Following similar lines, Metchnikoff directed the performance of experiments which lead him to believe that lactic acid-producing bacilli once successfully established in the intestinal tract decrease or even prevent the multiplication of putrefactive organisms. This inhibiting effect he attributes to the lactic acid which is produced by microorganisms introduced in these experiments.

Where putrefactive disturbances are already present the lactic acid bacilli appear to reenforce the enfeebled action of the normal intestinal lactic acid bacilli and increase the amount of acid produced where it is insufficient, or reintroduce it where it is absent, thus assisting the host to throw off the "wild races" of bacteria that may have become habituated to the intestinal tract. This, at least, is the assumption.

Not all lactic acid-producing bacteria are suitable for this purpose and the choice of a culture should be based upon the following criteria: First, the organisms should be able immediately or in time to become habituated to the intestinal tract; second, they should produce no toxins or putrefactive substances or other injurious products, detrimental to the host; third, they should be able to make sufficient lactic acid to accomplish the purpose for which they are introduced.

The organism which Metchnikoff selected for his researches was originally obtained from the Bulgarian ferment called "Yogourt" and the bacillus which apparently is the most potent lactic acid producer in this ferment has been named *B. bulgaricus*.<sup>1</sup> This organism, as we have studied it, is rather long ( $1 \times 4-6$  microns), large, with rounded ends, growing singly or in pairs, rarely in chains. It stains well with the ordinary anilin dyes particularly in young cultures, and is Gram-positive. In old milk cultures some of the rods may be Gram-negative while others present a punctate appearance, due apparently to the concentration of protoplasm in certain portions of the cell which are Gram-positive, and suggest Ernst-Babes granules, the remaining por-

<sup>1</sup> Cohendy: *Compt. rend. de la soc. de biol.*, ix, 1906.



tions undergoing rarefaction of protoplasm and becoming Gram-negative. On media containing no carbohydrates there is no growth. On dextrose or lactose agar the growth is slight and usually appears as small, stellate colonies rarely exceeding one to one and one-half millimeters in diameter. In stab cultures on the same media a slight growth appears after three to five days but always remains limited to the line of inoculation. In dextrose and lactose bouillon there is usually a feeble growth after several days, appearing as a fine sediment visible only after agitating the tube. In milk cultures, on the contrary, the growth is very vigorous, resulting in a rather finely flocculent coagulum with a minimal separation of fluid. In this medium considerable amounts of lactic acid are formed. Bertrand and Weissweiller<sup>1</sup> studied the chemical action of *B. bulgaricus* on milk and found that a slight amount (usually less than 10 per cent), of the casein is peptonized and apparently utilized as food by the bacterial cells. A small amount of the fat is saponified while practically all of the lactose is changed to dextro- and lævo-lactic acid, the dextro variety predominating. Twenty-five grams per liter of lactic acid are easily formed and at the same time small amounts of acetic, formic and succinic acids are produced, usually not more than half a gram per liter. Inoculations into milk are active, even after fourteen days, although at the end of three weeks the bacilli are usually dead. In our experience the amount of lactic acid produced by *B. bulgaricus* is sufficient at the end of forty-eight hours to render the milk unpleasant to the taste, particularly if previous to inoculation the culture has been frequently transplanted, so that the organisms are in an active vegetative state. Soured milk prepared according to Metchnikoff's directions is said to contain about ten grams of lactic acid per liter.<sup>2</sup>

Several investigations have been made by various observers to determine the effects of the Bulgarian bacillus upon the intestinal flora and various putrefactive products. Cohendy,<sup>3</sup> experimenting upon himself, found that pure cultures of lactic acid bacilli had a tendency to reduce intestinal putrefaction and that the

<sup>1</sup> *Ann. de l'inst. Past.*, xx, pp. 977-990, 1906.

<sup>2</sup> Metchnikoff: *The Prolongation of Life*, p. 180.

<sup>3</sup> *Loc. cit.*

organisms could be recovered from the feces without difficulty several weeks after the experiment was stopped. He took *B. bulgaricus* for about two and one-half months. Pochon<sup>1</sup> consumed cultures of lactic acid bacilli in milk and noted the diminution of indol and phenol in his feces. Leva<sup>2</sup> investigated the effect of Lactobacilline, milk, and milk plus Lactobacilline upon the excretion of ethereal sulphates, volatile fatty acids, aromatic oxyacids, phenol and indican. The experiment was divided into four periods: (1) a uniform daily diet; (2) diet + Lactobacilline;<sup>3</sup> (3) diet + Lactobacilline + one liter of milk; (4) diet + one liter of milk. His conclusions are as follows:

(1) The excretion of ethereal sulphates during the experiment was practically unchanged.

(2) The excretion of volatile fatty acids with Lactobacilline alone, or milk alone, as well as with Lactobacilline and milk combined, showed a considerable decrease.

(3) The excretion of aromatic oxyacids and hippuric acid was uninfluenced by milk, decreased distinctly in amount with Lactobacilline, decreased greatly with Lactobacilline + milk.

(4) The phenol excretion decreased somewhat under the influence of Lactobacilline alone, as well as with milk alone; there was a much greater decrease with a combination of Lactobacilline + milk.

(5) The indican excretion was very slight at the beginning of the experiment (too small an amount to measure accurately) and remained practically unchanged throughout the entire period.

Belonowsky<sup>4</sup> studied the influence of these organisms upon the intestinal flora of mice. His method was to contaminate the food (usually grain previously sterilized by heat) with *B. bulgaricus*, allowing the animals to eat sufficient quantities to make certain that the organisms were actually introduced in large numbers. His results may be summarized as follows: First, the Bulgarian

<sup>1</sup> Cited by Combe: *L'autointoxication intestinale*, Paris, 1906.

<sup>2</sup> Leva, J.: Zur Beurteilung der Wirkung des Lactobacillins und der Yoghurthmilch, *Berl. klin. Wochenschr.*, xlv, pp. 922-924, 1908.

<sup>3</sup> The Lactobacilline was obtained from "Le Ferment" Company of Paris. Leva's observation that a yeast was present in the Lactobacilline agrees with our own observation on this point. (See Fig. 1.)

<sup>4</sup> *Ann. de l'inst. Past.*, xxi, p. 991, 1907.

ferment modifies the normal intestinal flora of mice by a general alteration in their character and by elimination of putrefactive forms. There is a diminution in the total number of bacteria as well as a lessened virulence of the feces when these are introduced intraperitoneally or subcutaneously into other animals. Second, the action is not attributable to the formation of lactic acid alone, but also to certain products inhibitory in nature, formed by the bacilli themselves. Third, the organisms become more or less established in the intestine about the tenth day in adult mice and persist without further reinoculation for a considerable but variable interval of time. Fourth, the cultures seem to have exerted a beneficial action upon the mice, particularly on those infected with the organisms of mouse typhus; in this case the results are due exclusively to the lactic acid.

From these investigations it would appear that many of the animals fed upon grain contaminated with the Bulgarian ferment gained in weight, that the feces contained fewer organisms capable of growing upon ordinary culture media, that putrefactive organisms tended to disappear and that this beneficial action was due in part to the lactic acid, in part to the products of metabolism of the bacteria themselves. The fact that relatively few of the Bulgarian bacilli are microscopically discernible in the feces raises the question, In what portion of the intestinal tract do these bacilli find their most favorable environment? If they occur in the upper (duodenal or jejunal) regions of the small intestine and only a few gain a foothold in the large intestine (usually considered the chief site of putrefaction) the organisms must act from a distance and their products, theoretically at least, must be less effective than if they were generated at the focus of infection. No experiments so far have been recorded which answer this question and the present investigation has approached the problem from this point of view. For our work, which was undertaken specifically to study the distribution of *B. bulgaricus* in the intestinal tract, the preparation called *Bacillac* was employed. This is said to be made according to Metchnikoff's personal directions, cultures of the organism described above (*B. bulgaricus*) being employed for this purpose. This organism is stated to have been isolated by Metchnikoff. The *Bacillac* is obtainable in pint bottles and contains a moderately large, Gram-positive

bacillus and (by accident or design) a large, oval, Gram-positive yeast as well (see Fig. 1). The bacillus isolated from specimens of *Bacillac* grows slowly upon ordinary dextrose and lactose agar, very poorly in corresponding bouillon media, but luxuriantly in milk. It produces in the latter medium a soft coagulum which after standing for a few days becomes massed into more or less permanent lumps with a moderate separation of fluid. The acidity increases rapidly and after forty-eight hours becomes decidedly unpleasant to the taste.

Partly because of its rapid growth, but chiefly because of the considerable amount of acid which this bacillus produces, it is easy to obtain cultures of the organism grown in milk at 37° C., even if it be originally associated with other organisms. Careful sub-culturing gives a differential enrichment of the Metchnikoff bacillus, so that one may obtain it in pure culture, as may be demonstrated by plating on slightly acid Bierwort agar. In the present investigation this method of enrichment has been used successfully for the isolation of the organism from the mixed intestinal flora.

For experimental purposes a moderate sized Rhesus monkey was used. The animal received daily half a liter of sweet milk for a period of three days. The feces were examined for lactic acid<sup>1</sup> as well as for organisms resembling the Metchnikoff bacillus

<sup>1</sup> Fletcher and Hopkins: *Journ of Physiol.*, xxxv, pp. 247-309, 1908.

Reagents:

- (1) Very dilute alcoholic solution of thiophene (10 to 25 drops in 100 cc.
- (2) Saturated aqueous solution of copper sulphate.
- (3) Concentrated sulphuric acid.

Procedure: 5 cc. strong sulphuric acid, 1 drop copper sulphate and a few drops of the suspected mixture are well shaken, then heated for 2 to 5 minutes in a water-bath in a test tube. Cool the solution, add 2 to 3 drops thiophene solution, replace in water-bath and again heat, watching constantly. Lactic acid rapidly and characteristically gives a bright cherry-red color under these conditions. The lactic acid must be as nearly free from water and organic matter as possible (malic acid and probably other oxy-acids give the reaction).

The lactic acid may be employed as an alcoholic solution or as a syrupy residue. Acetaldehyde and glyoxylic acid give color reactions with thiophene and sulphuric acid, but the copper sulphate used as above destroys

either culturally or morphologically. In no instance was lactic acid found.

The experiment with *Bacillac* was carried out as follows. The amount of soured milk given was the same as in the control observations, namely, five hundred cubic centimeters daily. At the end of the second day, chemical and bacteriological examinations were commenced, but until the sixth day no lactic acid bacilli were isolated nor could lactic acid be detected in the animal's feces. Two days later the first positive test for lactic acid was obtained, using the thiophene reaction of Fletcher and Hopkins. The feces were slightly but distinctly acid at this time—more acid than upon previous occasions. It was difficult to obtain a satisfactory test for lactic acid from the feces owing to the presence of a brownish-yellow coloring matter soluble in ether. But it was possible by removing the ether through evaporation, taking up the oily residue in water, boiling with animal charcoal, filtering, washing, and again evaporating to a syrup to obtain a slightly yellow solution which gave a good reaction with the thiophene. Controls made from normal feces of the same animal (free from lactic acid), which were treated in the same manner, but to which were added known minute amounts of lactic acid, gave in every instance the same color reaction. After fourteen days the monkey was given the usual meal of five hundred cubic centimeters of *Bacillac*. Then, after allowing three and one-half hours for digestion (the whole portion of milk having been consumed at this time) the animal was killed by chloroform and examined. Samples taken from the stomach and from various levels of the small and large intestines were removed with appropriate precautions for microscopical, bacteriological and chemical examination. The material for chemical examination was placed in ether slightly but distinctly acidified with sulphuric acid. The bacterial material was inoculated into milk and fermentation tubes containing dextrose, lactose and saccharose bouillon. The specimens for microscopical study were smeared on slides and stained by the Gram method for identification of forms resem-

them. If ether is employed for extracting lactic acid, it should be first washed with water to remove aldehyde bodies. The color produced by lactic acid is transitory unless the tube be cooled immediately after its appearance.

bling morphologically the acid bacillus fed. The animal was in good health before and during the experiment. At autopsy about one hundred cubic centimeters of partially digested milk were found in the stomach. The small intestine contained a moderate amount of semi-fluid, yellowish, gelatinous substance. At the ileo-cæcal valve the contents were more abundant and about the consistence of thick paste. The color was a deeper brown. The color and consistence increased progressively to the anus, where the feces were solid and fairly dark. The mucous membrane throughout the gastro-intestinal tract appeared normal, although the reaction of the contents from the stomach to the anus was distinctly acid to litmus.

The Bulgarian bacilli were present and easily demonstrated by smears and by cultures in relatively large numbers in the stomach contents but associated with yeasts. In some instances, particularly where the milk was obviously undergoing digestion, the organisms showed undoubted signs of degeneration. The staining was very irregular and faint in such bacilli, whereas the yeasts, so far as could be determined by microscopical examination, showed no such changes. In the duodenum and ileum the Bulgarian organisms were encountered in almost pure culture, although inoculations into fermentation tubes showed a few gas-forming bacilli of the colon type. In the region of the ileo-cæcal valve there was a rather abrupt change in the nature of the bacterial smears. Not only was the amount of fecal material greater but the character of the microorganisms was different. *B. bulgaricus* ceased to be the dominant organism, although it was still present in moderate numbers. Gas-forming bacteria were, on the other hand, increased enormously. Gram-positive rods and cocci were also present. No attempt was made to identify the latter. From the ileo-cæcal valve, progressively down the large intestine towards the anus, the number of the Bulgarian bacilli decreased while the number of other bacteria increased, until at the rectum there were very few Metchnikoff bacilli but enormous numbers of bacteria of the colon type and many Gram-positive rods.

Lactic acid was demonstrated throughout the gastro-intestinal tract as well as in the feces. Although no attempt was made to determine quantitatively the amount of acid at any level of the

intestine, the results indicated that much less lactic acid was present in the large intestine than in the stomach and small intestine. This diminution (shown by the decidedly lessened color developed by the thiophene, using approximately equal amounts of intestinal contents) began rather abruptly at the region of the ileo-cæcal valve, and progressively increased to the anus. This phenomenon was particularly marked in the lower portions of the large intestine, where the contents were more desiccated. The amount of material obtained from this region was greater than was the case in higher levels, while at the same time the volume of lactic acid was decidedly less, although the ether extraction was prolonged. This coincides with the relatively smaller number of lactic acid bacilli found.

#### CONCLUSIONS.

(1) By feeding a Rhesus monkey for two weeks exclusively on milk fermented with *B. bulgaricus* (but containing also some yeasts) it was possible to maintain an acid reaction throughout the digestive tract. The acid reaction was more pronounced above the ileo-cæcal region than at this region or below it. The acidity decreased progressively from the ileo-cæcal region to the anus. Lactic acid was detectable at every point in the digestive tract that was tested, the reaction growing less marked below the ileo-cæcal region.

(2) Exclusive feeding for two weeks with milk fermented with *B. bulgaricus* failed to establish the predominance of this organism in the ileo-cæcal region or in the large intestine. In the latter situation the number of bacilli of this type was relatively small and decreased towards the anus. Thus in the regions characterized by most active putrefaction the lactic acid bacilli failed to establish themselves in relatively large numbers.

## DESCRIPTION OF THE PLATES.

Fig. I. Smear from stomach contents of monkey, three and one-half hours after feeding *Bacillac*. Practically pure culture of *B. bulgaricus* and few yeast cells. Bacteria show "punctate" staining due, apparently, to combined effect of partial digestion and excessive acidity.

Fig. II. Contents of small intestine in the region of the duodenum. The Bulgarian bacilli predominate. A few punctate forms are seen. The bacteria are multiplying at this point. The normal appearance of the bacteria is in accordance with this observation.

Fig. III. Contents of the small intestine near the ileo-cæcal valve. Uniformly staining Bulgarian bacteria are present, but not in predominating numbers as in Fig. II. Gram-negative forms begin to predominate.

Fig. IV. Contents of the large intestine about two feet from the ileo-cæcal valve. A few typical Bulgarian bacilli still persist. One of these shows the punctate staining seen in the stomach. Yeast cells are also present. Gram-negative bacilli are the dominant forms.

Fig. V. Feces of monkey. Bulgarian bacilli have practically disappeared. They were, however, readily demonstrated by the milk-enrichment method described in the text.

Fig. VI. Fermentation sediment obtained from a lactose fermentation-tube inoculated with intestinal contents obtained from the level of the ileo-cæcal valve (cf. Fig. III). No Bulgarian bacilli are present in this sediment, although they were readily demonstrated in the smear made at this level as well as culturally in milk. (This figure is introduced to show that the Bulgarian bacilli do not grow in fermentation media.)

The slides from which these plates are reproduced were stained by Gram's method. Magnification, 1000 diameters. Zeiss 2 mm. homo. imm. apochromatic lens.

Our thanks are due to Dr. Leaming of the Rockefeller Institute for Medical Research for the above photographs.





Fig I. Smear from stomach contents of monkey, showing great predominance of *B. Bulgaricus* and a few yeast cells.



Fig. II. Smear from contents of duodenum: *B. Bulgaricus* predominating.



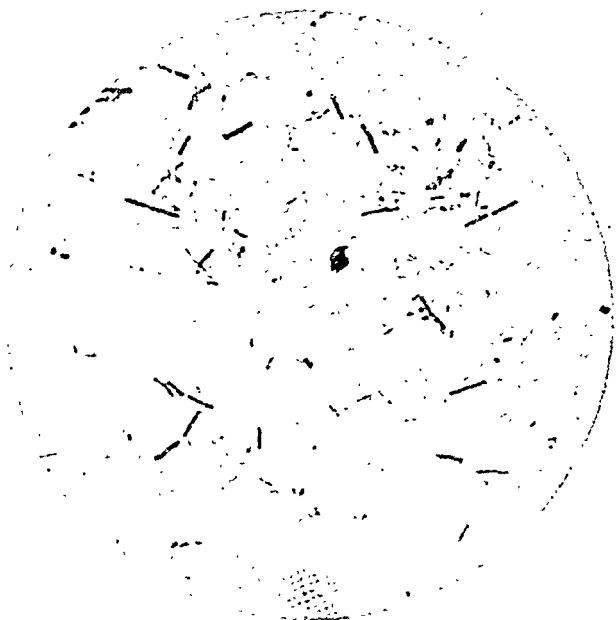


Fig. III. Smear from small intestine: *B. Bulgaricus* prominent but not predominating.

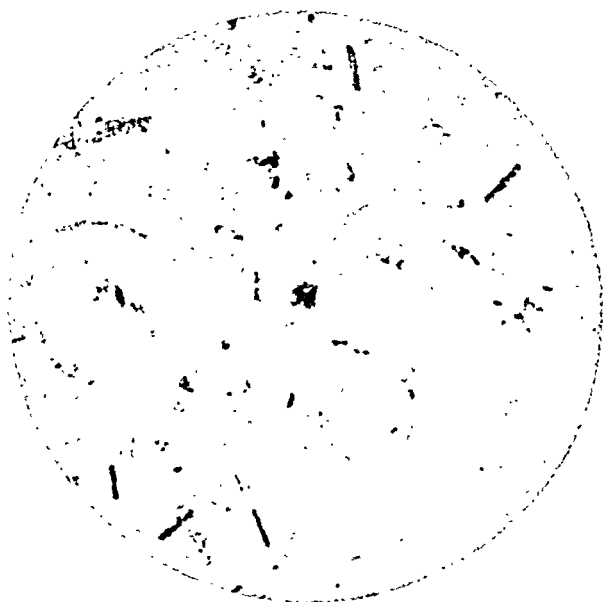


Fig. IV. Smear from large intestine: *B. Bulgaricus* much less prominent than in regions above.



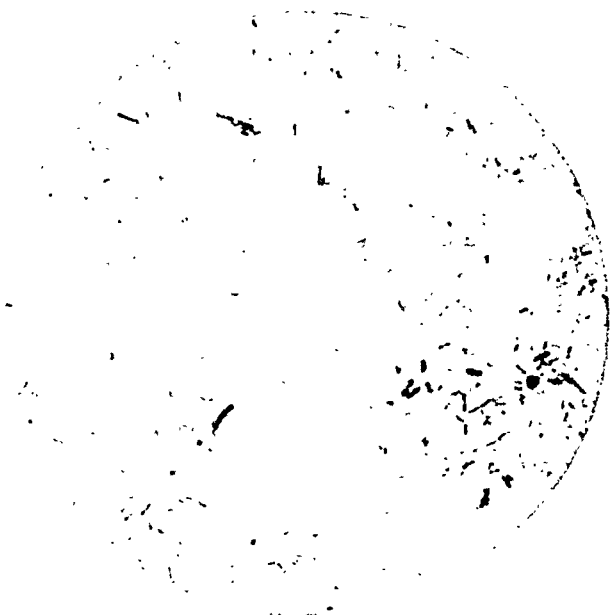


Fig. V. Smear from feces: *B. Bulgaricus* has almost disappeared.

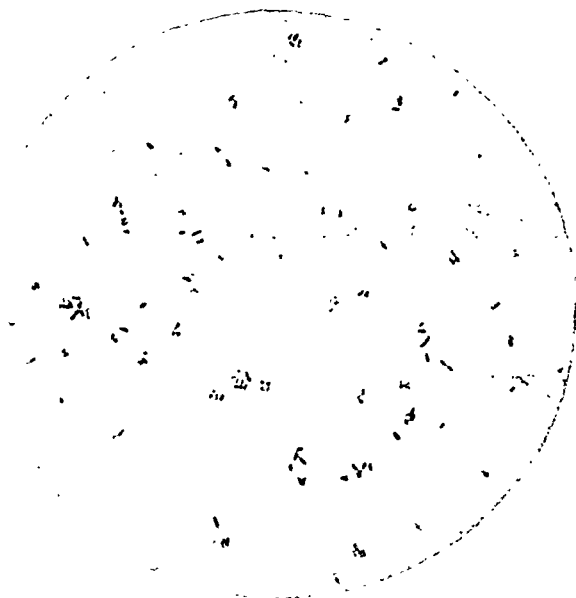


Fig. VI. Smear from fermentation-tube sediment from lactose fermentation-tube inoculated with intestinal contents from the level of the ilco-cæcal valve.



# FURTHER STUDIES OF THE MODE OF OXIDATION OF PHENYL DERIVATIVES OF FATTY ACIDS IN THE ANIMAL ORGANISM. III.

(SYNTHESIS OF SOME DERIVATIVES OF PHENYLPROPIONIC ACID.)

By H. D. DAKIN.

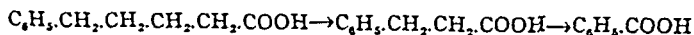
(From the Laboratory of Dr. C. A. Herter, New York.)

(Received for publication, October 20, 1908.)

In a former communication<sup>1</sup> it was stated that cinnamoyl-glycocoll had been identified as an intermediary product in the catabolism of phenylpropionic acid and phenylvaleric acid. This result suggested much that is of interest not only with regard to the mode of catabolism of fatty acids, but also as to the origin of the unsaturated fatty acids in the body and in addition, the rôle which glycocoll and other amino-acids play in fatty acid metabolism.

Knoop's observation<sup>2</sup> of the excretion of hippuric acid following the administration of phenylvaleric acid to dogs left the question open as to whether the side-chain had been oxidized directly in the  $\delta$ -position or not, but the observation of the formation of cinnamoyl-glycocoll at once proves that, at least in part, the oxidation takes place at the  $\beta$ -carbon atom with removal of two carbon atoms and that a second oxidation in the  $\beta$ -position results in the formation of benzoic or rather hippuric acid. These results furnish a complete proof of the accuracy of the hypothesis of  $\beta$ -oxidation advanced in the first instance by Knoop.

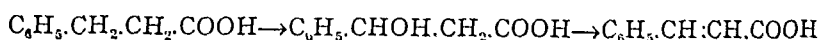
Excluding the intermediary steps in the oxidation, and without representing the glycocoll grouping, the change may be expressed as follows:



<sup>1</sup> This *Journal*, v, p. 185 (Note added during proof correction).

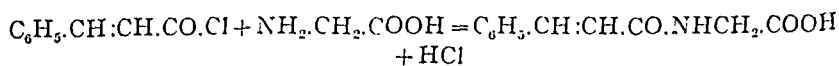
<sup>2</sup> *Beitr. z. chem. Physiol. u. Pathol.*, vi, p. 150, 1904.

The formation of cinnamoylglycocoll following the administration of phenylpropionic acid can apparently only be explained on the assumption of a prior formation of phenyl- $\beta$ -oxypropionic acid or a derivative of it, which subsequently parts with a molecule of water to give cinnamic acid or a derivative of cinnamic acid:



It is a curious fact, however, that phenyl- $\beta$ -oxypropionic acid *itself*, administered to an animal, proves to be far more difficult of combustion than phenylpropionic acid, the acid being excreted largely unchanged and not combined with glycocoll. It would therefore appear as if combination with glycocoll might be a necessary preliminary to oxidation. To test this hypothesis it was necessary to synthesize the glycocoll derivative of phenyl- $\beta$ -oxypropionic acid and to determine its behavior in the body. The following paper contains an account of the synthesis of this substance together with the synthesis of cinnamoylglycocoll and some of its derivatives. The synthetic cinnamoylglycocoll was found to be identical in every respect with the substance isolated from the urine of animals that had received injections of sodium phenylpropionate or sodium phenylvalerate.<sup>1</sup>

Cinnamoylglycocoll was prepared from cinnamic acid which was converted in the acid chloride and the latter substance was allowed to interact with glycocoll in the presence of caustic soda, at a low temperature:

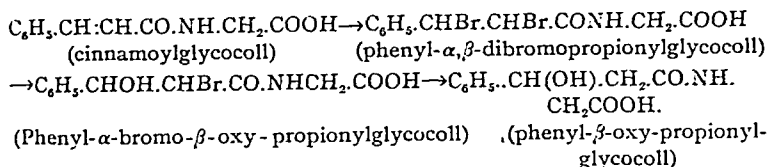


Apart from its behavior as an unsaturated substance, cinnamoylglycocoll resembles hippuric acid closely. On reduction with sodium amalgam it is converted into phenylpropionylglycocoll.

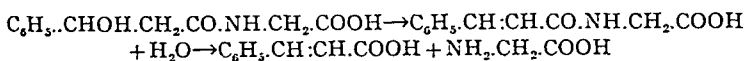
Cinnamoylglycocoll on bromination in acetic acid solution is converted into phenyl- $\alpha,\beta$ -dibromopropionylglycocoll which on boiling with water is converted into phenyl- $\alpha$ -bromo- $\beta$ -oxy-propionylglycocoll and this substance on reduction with sodium amalgam yields the desired phenyl- $\beta$ -oxy-propionylglycocoll.

<sup>1</sup> An account of the animal experiments will be published shortly.

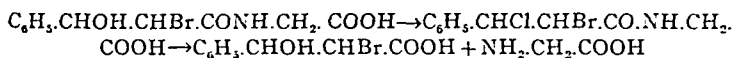




Phenyl- $\beta$ -oxypropionylglycocoll on heating with concentrated hydrochloric acid, parts with the element of water to yield cinnamoylglycocoll.<sup>1</sup> On further heating the latter undergoes hydrolysis with formation of cinnamic acid and glycocoll.



Phenyl- $\alpha$ -bromo- $\beta$ -oxypropionylglycocoll on boiling with concentrated hydrochloric acid is converted primarily into an almost insoluble substance, which proved to be phenyl- $\alpha$ -bromo- $\beta$ -chloro-propionylglycocoll. On further heating with more dilute acid phenyl- $\alpha$ -bromo- $\beta$ -oxypropionic acid is obtained. These reactions afford convincing evidence of the correctness of the constitutions assigned to the various substances.



#### EXPERIMENTAL PART.

##### *Synthesis of Cinnamoylglycocoll.*

Cinnamoylchloride was prepared in the usual way by acting upon cinnamic acid with phosphorus pentachloride, distilling off the phosphorus oxychloride and fractionating the residue *in vacuo*. The acid chloride quickly solidifies and melts at 35° to 36°. The cinnamoylchloride (16.6 grams) was melted and slowly dropped into a flask containing 8.0 grams of glycocoll dissolved in 80 cc. of 10 per cent caustic soda solution. The flask was kept in a freezing mixture and a few cubic centimeters of ether were added in order to assist in dissolving the cinnamoylchloride. The contents of the flask were shaken vigorously until all the

<sup>1</sup> This reaction is of interest since formation of cinnamoylglycocoll in the body probably results from a similar change.

cinnamoylchloride had disappeared. The clear solution was then acidified with sulphuric acid and the precipitate of crude cinnamoylglycocoll was filtered off, washed with cold water, dried and then washed with a little ether to remove a trace of cinnamic acid. The cinnamoylglycocoll was recrystallized from boiling water and separated in the form of long shining needles melting sharply at  $192^{\circ}$  to  $193^{\circ}$ . The yield of recrystallized substance was 15 grams, equivalent to about 75 per cent of the theoretical amount.

#### ANALYSIS.

0.2005 gm. gave 0.01358 gm. N. = 6.77 per cent N.  
 $C_{11}H_{11}NO_3$  requires 6.83 per cent N.

Cinnamoylglycocoll is sparingly soluble in cold water and moderately soluble in boiling water. It is easily soluble in alcohol and ethyl acetate but almost insoluble in dry ether, chloroform and petroleum ether. It is a fairly strong acid and readily forms salts. When dissolved in a little sodium carbonate solution, it instantly reduces dilute potassium permanganate and an odor of benzaldehyde is at once noticeable. On boiling with strong hydrochloric acid it is reconverted into cinnamic acid and glycocoll.

The synthetic cinnamoylglycocoll was identical in every way with that isolated from the urine of animals that had received injections of sodium phenylpropionate or sodium phenylvalerate and the melting point of a mixture of the substances of different origin was unchanged.

#### *Reduction of Cinnamoylglycocoll.*

Cinnamoylglycocoll, 0.5 gram, was suspended in 10 cc. of water and treated with 20 grams of 2 per cent sodium amalgam. After standing for a couple of hours in a warm place the solution was precipitated with hydrochloric acid. 0.35 gram of phenylpropionylglycocoll, m.p.  $114^{\circ}$ , was obtained. The substance was identical with that previously prepared from phenylpropionylchloride and glycocoll.<sup>1</sup>

<sup>1</sup> This *Journal*, iv, p. 431, 1908.

## ANALYSIS.

0.2865 gm. gave 0.01932 gm. N (Kjeldahl) = 6.74 per cent N.  
 $C_{11}H_{13}NO_3$  requires 6.76 per cent N.

*Synthesis of Phenyl- $\beta$ -oxy-propionylglycocoll.*

PHENYL- $\alpha,\beta$ -DIBROMOPROPIONYLGLYCOCOLL. Cinnamoylglycocoll (10.25 grams) was dissolved in 60 cc. of warm glacial acetic acid. The solution was cooled to a point just short of crystallization and then bromine (8.0 grams) dissolved in 15 cc. of glacial acetic acid was added fairly rapidly. While the bromine was being added, the liquid was well shaken and cooled under the tap. The bromine was rapidly absorbed and after a few moments the solution was diluted with ice water and the precipitated phenyl- $\alpha,\beta$ -dibromopropionylglycocoll filtered off and washed with cold water. The yield is practically quantitative. The substance when heated rapidly melts with complete decomposition at  $190^\circ$  to  $191^\circ$ . If heated slowly it decomposes at a slightly lower temperature. It is very sparingly soluble in ether and in cold water and insoluble in carbon bisulphide, chloroform and petroleum ether. It is readily soluble in glacial acetic acid and crystallizes in hard, shining prisms.

## ANALYSIS.

0.2045 gm. gave 0.2134 gm. AgBr = 44.4 per cent Br.  
0.2621 gm. gave 0.00987 gm. N (Kjeldahl) = 3.77 per cent N.  
 $C_{11}H_{11}Br_2O_3N$  requires 3.84 per cent N and 43.7 per cent Br.

PHENYL- $\alpha$ -BROMO- $\beta$ -OXYPROPIONYLGLYCOCOLL. Phenyl- $\alpha,\beta$ -dibromopropionylglycocoll readily parts with one atom of bromine when boiled with water.<sup>1</sup> Five grams of the substance were boiled with 75 cc. of water under a reflux condenser until all the acid had dissolved. The solution was then extracted with ether in a continuous extraction apparatus for three hours. On evaporation of the ether an almost quantitative yield of phenyl- $\alpha$ -bromo- $\beta$ -oxypropionylglycocoll was obtained. It crystallizes in needles and is readily soluble in water, alcohol and ether. It may conveniently be recrystallized from water and melts at  $87^\circ$  to  $88^\circ$ .

<sup>1</sup> An estimation of the amount of bromine liberated as hybromic acid on boiling with water gave 22.7 percent Br, theory demanding 21.92 per cent.

## ANALYSIS.

0.2274 gm. gave 0.1380 gm. AgBr = 25.82 per cent Br.

0.1672 gm. substance dried at 70° gave 0.00749 gm. N (Kjeldahl) = 4.49 per cent.

0.2194 gm. substance dried at 70° gave 0.01008 gm. N (Kjeldahl) = 4.59 per cent.

$C_{11}H_{12}O_4$  NBr requires 4.63 per cent N and 26.48 per cent Br.

Phenyl- $\alpha$ -bromo- $\beta$ -oxypropionylglycocoll when covered with concentrated hydrochloric acid and gently warmed under a reflux quickly dissolves and after a few moments a copious separation of a fine crystalline substance takes place. This substance is very sparingly soluble in water and crystallizes in hard, highly refractive hexagonal prisms which melt at 203° to 204° with complete decomposition. On analysis the substance proved to be phenyl- $\alpha$ -bromo- $\beta$ -chloropropionylglycocoll, the alcoholic hydroxyl group of the oxy-acid having been replaced by chlorine.

## ANALYSIS.

0.1565 gm. gave 0.1602 gm. AgCl, AgBr.

Calculated for  $C_{11}H_{11}O_3NCIBr$  = 0.1622 gm.

0.1896 gm. gave 0.0084 gm. N (Kjeldahl) = 4.43 per cent.

$C_{11}H_{11}O_3NCIBr$  requires 4.38 per cent.

On boiling with water the substance slowly dissolves with liberation of hydrochloric acid and phenyl- $\alpha$ -bromo- $\beta$ -oxypropionylglycocoll, m.p. 87° to 88°, crystallizes out on cooling. The substance is almost insoluble in ether but readily dissolves in alkali and its aqueous solution does not decolorize alkaline permanganate in the cold.

If instead of filtering off the insoluble precipitate, of phenyl- $\alpha$ -bromo- $\beta$ -chloropropionylglycocoll the boiling with hydrochloric acid is continued, the precipitate slowly dissolves, especially if the acid be diluted, and eventually oily drops appear. On cooling the oil drops solidify and on recrystallization from a mixture of chloroform and petroleum, phenyl- $\alpha$ -bromo- $\beta$ -oxypropionic acid is obtained in the form of crystals melting at 125° to 126°.

PHENYL- $\beta$ -OXYPROPIONYLGLYCOCOLL. The remaining bromine atom in phenyl- $\alpha$ -bromo- $\beta$ -oxypropionylglycocoll is readily replaced by hydrogen when treated with sodium amalgam in

faintly acid solution. The substance (5.0 grams) was dissolved in twenty parts of water and treated with three times the theoretical amount of 2.5 per cent sodium amalgam. The solution was kept acid by the occasional addition of a little sulphuric acid. After some hours the solution was strongly acidified with phosphoric acid and extracted with ether containing 10 per cent alcohol in a continuous extraction apparatus. On distilling off the ether an almost quantitative yield of phenyl- $\beta$ -oxypropionylglycocoll was obtained. The substance is easily soluble in water and alcohol and crystallizes from a mixture of alcohol and chloroform or from water in star-shaped aggregations of needles, m.p.  $146^{\circ}$  to  $147^{\circ}$ .

## ANALYSIS.

0.1402 gm. gave 0.00875 gm. N (Kjeldahl) = 6.23 per cent N.  
 $C_{11}H_{13}O_4N$  requires 6.28 per cent N.

Phenyl- $\beta$ -oxypropionylglycocoll dissolved in sodium carbonate solution gives benzaldehyde when gently warmed with potassium permanganate. On heating to boiling with strong hydrochloric acid and at once cooling, a precipitate of cinnamoylglycocoll is obtained, which crystallizes from water in long needles and melts at  $192^{\circ}$  to  $193^{\circ}$ . On prolonged heating with hydrochloric acid cinnamic acid, m.p.  $133^{\circ}$  is obtained.



## CHEMICAL STUDIES IN CYTOLYSIS.

By ALONZO ENGLEBERT TAYLOR.

(From the Hearst Laboratory of Pathology, University of California.)

(Received for publication, October 10, 1908.)

The phenomenon of spermatolysis, first described by Metchnikoff, offers in many respects favorable opportunities for the study of the details and variables of the reaction of cytolysis. The following report represents the results of an investigation designed to elucidate the nature of the active factor or constituent in the spermatozoön which, on inoculation into an appropriate animal, gives rise to the formation there of the antibody which acts as the spermolytic agent when the blood of the immunized animal is mixed with the spermatozoa under consideration. When the living spermatozoa (or fresh dead sperm) of the salmon (*Oncorhynchus Quinnet*) are injected into a rabbit, the blood of the animal acquires the power of causing cytolysis of salmon spermatozoa. The reaction of cytolysis, under the microscope, is resolved into two stages. First the spermatozoa swell; later the cell membrane seems to dissolve or burst, and the protoplasm becomes converted into a detritus. The first step, swelling, is not essential to the cytolysis. Whenever these cells are placed in a salt solution of the concentration of the serum of the rabbit (that of the spermatid fluid is much lower in osmotic pressure) they swell. If the serum be an immune one, in addition to the swelling, the cell wall dissolves or bursts. This swelling of these cells when placed in salt solution is not a phenomenon peculiar to these cells, but is one of widespread occurrence. It can be demonstrated in a striking manner, macroscopically, by placing ripe salmon eggs in a bottle in a strong solution of sodium chloride. It might at first be supposed that under such circumstances salt would pass into the eggs and water pass out, that a shriveling of the eggs would occur; in fact, the eggs swell. This swelling is in all probability due to

the passage of both salt and water into the eggs, the membrane does not act as the dividing membrane in an osmosis cell. The swelling of the spermatozoa in a serum, normal or immune, has therefore no relation to the cytolysis. This can be proved directly by mixing the active serum with three parts of distilled water, when the suspended spermatozoa will undergo cytolysis without swelling.

To what is this cytolysis of the spermatozoa due? There can be no purpose in a review here of the now enormous literature on the subject of specific cytolysis. Two generalized views are current; one that it is due to a disturbance of the complex lipid-protein in the cell membrane, whereby the integrity of the same is destroyed; the second is that it is due to a disturbance of the complex lipid-protein in the protoplasm. That the lipid fraction is essential to the physical constitution of the protoplasm as well as of the cell membrane seems quite certain. It would be easy to say that in cytolysis the colloidal nature of the protoplasm or membrane is reduced or destroyed; but little would be gained in the concrete sense by such an expression. As is well known, a fractionation of red corpuscles has led to the conclusion that it is the lipid fraction which on injection gives rise to the activation of the serum of the animal receiving the injections. Since salmon spermatozoa may be obtained at the hatcheries in large quantities, one may attempt upon a large scale the fractionation of these cells in the chemical sense; and attempt to identify and isolate the substance or fraction that on injection into the rabbit causes the formation of the antibody there.

Something over two kilos of salmon sperm was submitted to such a fractionation. In view of the known chemical composition of the spermatozoa, due largely to the researches of Miescher and Kossel, the cells were divided into four fractions: (a) the protamin; (b) the nucleinic acid; (c) the lipoids soluble in ether by simple extraction; and (d) the lipoids soluble in ether after the digestion of the residue of (c) with trypsin. Rabbits were inoculated for three weeks with ascending doses of *a*, *b*, *c* and *d* and with mixtures of *c* and *d* suspended in isotonic salt solution; also with fresh spermatozoa. Protamin is quite toxic to rabbits; the nucleinic acid causes a leucocytosis, but is



otherwise not toxic in ordinary doses; the lipoids are not toxic in ordinary doses. Fresh spermatozoa of the salmon are surprisingly toxic to rabbits, and several deaths were due to these injections. This toxicity is not a matter of salts, isotonicity or mass of the injection fluid; it is resident in the sperm. The sera of the several inoculated animals were collected in the usual manner, conserved upon ice and taken to the hatchery and tested upon matured freshly recovered spermatozoa.

Except in the case of the sera from rabbits inoculated with the spermatozoa the results were entirely negative. The active sera obtained from the animals inoculated with the sperm were not, however, highly active; a dilution of 10 : 1 was sufficient to obliterate the cytolytic action. Curious is the fact that unless the spermatozoa be fresh, no cytolysis occurs. If the sperm has been out of the body of the fish for an hour, the spermatozoa can no longer be cytolized by a serum that is effective with the freshly removed spermatozoa of the same fish. One may infer that within this hour a coagulation-necrosis has occurred within the cells. Entirely inactive, as stated, were the sera obtained from the animals inoculated with the lipoidal, protamin and nucleinic acid fractions; swelling of the spermatozoa occurred, but no cytolysis.

It cannot be taken as proved by these experiments that the activation of a serum is not due to a chemically definable substance in the cells employed; that it is, to use a poor but expressive phrase, not a chemical but a biological fraction that produces the antibody in the animal receiving it. It is possible that the period of inoculation was not long enough. This is, however, very unlikely, since the period of inoculation with the sperm, which was effective, was much shorter and concerned much smaller quantities. It might be that the materials were chemically altered in the course of the manipulations of isolation. This could surely not hold for the protamin and the nucleinic acids; it might, however, have been true for the lipoids. Lastly it might be assumed that not one substance alone is needed, but the interaction or combined action of a second substance is necessary. Thus, it might possibly be not the lipid but the complex lipid-protein that is necessary. Speculation upon this matter can lead to no result. This experiment proves only one

thing: that the ether-soluble fraction of the spermatozoa of the salmon, like the protamin and nucleinic acids, has not the power of causing in the inoculated animal the formation of an anti-body capable of producing cytolysis of the spermatozoa of the same fish.

To Mr. E. M. Ball, director of the Battle Creek Hatchery, California, I express my gratitude for permission to carry on the work and for the many acts of courtesy and assistance extended to me.

# ON THE CONVERSION OF GLYCOGEN INTO SUGAR IN THE LIVER.

By ALONZO ENGLEBERT TAYLOR.

(From the Hearst Laboratory of Pathology, University of California.)

(Received for publication, October 10, 1908.)

The older literature of physiology contains many papers dealing with the relation of the liver to the conversion of glycogen into sugar. The facts that sugar is known as a simple chemical reaction to be formed from glycogen, that the carbohydrates of the digestion pass on resorption into the portal circulation and not into the lacteal system, and that the liver contains glycogen in amounts that are proportionately related to the carbohydrates of the diet were sufficient to convince Claude Bernard that glycolysis is a function residing in the liver. The fact that analytically it has been difficult to demonstrate a greater sugar content in venous than in the arterial or portal blood (of the liver) proves nothing when it is realized that if in a dog of 10 kilos weight 100 grams of glycogen were daily converted into sugar in the liver, the amount of sugar passing out of the liver through the hepatic vein in each second of time would be a little over 1 milligram. The chemical demonstration of the relations is impossible in the higher animals. The reaction may, however, be easily studied upon invertebrate material, as the experiment reported in the following lines will illustrate. An obvious assumption in the argument is that facts determined upon such simple material are held to be valid in mammalian physiology.

The large clam of the Pacific Coast (*Schizothærus Nuttallii*) presents in the liver amounts of glycogen that may run as high as 8 per cent of the dried residue. The amount of sugar that is to be found in the same fresh material is low. These facts are known to hold only for the material collected at the very low tides, after the period of feeding. The livers of some twenty of these large bivalves were carefully freed of the other structures,

ground to a pulp, and an adequate amount of toluol added to prevent bacterial action. A known amount of the total mass was then placed in strong alcohol in the proportions of 1 : 3, and later subjected to analyses for sugar, glycogen and total nitrogen. From these figures the original substrate of the reaction experiment (glycogen) and the product (sugar) could be calculated for the total reaction system. The nitrogen was determined as a basis for the calculation of the amounts removed for the analyses of sugar at the stated times. The reaction material was placed in a jar and suspended into the water of a deep well whose temperature ( $12.4^{\circ}$ ) was nearly constant day and night, as determined by the thermometer introduced with the jar containing the material. From time to time known quantities were removed, mixed with three parts of alcohol, and later analyzed for total nitrogen, sugar and glycogen. With a thick mass of this kind, it is not possible to measure accurately the quantities removed, but by the determination of the total nitrogen and the comparison of this figure with the figure for the total determined in the beginning of the experiment, the exact amount of material removed for analysis could be estimated. The sugar was estimated by the gravimetric method of Pflüger, the copper being weighed as the reduced metal; the glycogen was estimated by the last method of Pflüger; the nitrogen was estimated by the method of Kjeldahl. The data contain obviously the desiderata of a quantitative experiment. The original total mass was known, the original total nitrogen, glycogen and sugar. The several parts removed for analyses at the stated times were known and the sugar in them determined by an accurate method. The total concentration of the system was constant, constant also the temperature. Culture tests done at the close of the experiment showed the material to be sterile. From the analytical data the following constants were obtained by the use of the common equation representing the progress of a monomolecular reaction:

$$\frac{dx}{dt} = C (A - x)$$

in which  $A$  and  $x$  stand respectively for the substrate and the

product of the reaction—the glycogen and the sugar. The times given are in hours.

$T =$	4	8	12	16	26	28	32	38
$C (\times 10^{-4}) =$	70	79	74	48	66	58	58	55

From these constants it is obvious that corresponding to the gradually decreasing figures for the constants, the curve of reactions falls away somewhat from that representing accurately a monomolecular reaction. This fact, however, is exactly what is found in the digestion of starch or glycogen by amylase *in vitro*; and the figures obtained in this experiment deviate no more than these from the normal curve of a monomolecular reaction. This slowing of the reaction is in all probability due to a gradual inactivation of the ferment through hydrolysis; in terms of physical chemistry, the constants fall because the mass of the catalysor is reduced.

This experiment represents what is commonly called a post-mortem digestion. Since glycogen diffuses with the greatest difficulty, it is certain that the reaction occurs in the liver cells and not in the intercellular fluids. A postmortem digestion differs from the reaction *in vivo*, so far as known, simply in this: in the living, fasting animal, the sugar is removed from the liver as fast as formed, the mass of the product of the reaction is therefore practically nil; the mass of ferment is subject to variations through new formation by the liver cells. In the experiment *in vitro*, it can be shown that the mass of sugar present under the conditions of the experiment is of practically no action in retarding the progress of the reaction. To what extent variations in the mass of glycolytic ferment in the liver occur within the same period of time in a fasting animal, is not known. Under the circumstances, however, we are warranted in assuming a close analogy between the postmortem and the living hydrolysis of glycogen in the liver; and the conclusion is thus reached, that in the living animal the conversion of glycogen into sugar in the liver is a function of two variables and proportional directly to them, namely, the masses of glycogen and of glycolytic ferment in the liver cells.



## ON THE ANTAGONISM OF ALCOHOL TO CARBOLIC ACID.

By ALONZO ENGLEBERT TAYLOR.

*(From the Hearst Laboratory of Pathology, University of California.)*

*(Received for publication, October 10, 1908.)*

It is a common practice with surgeons to apply ethyl alcohol to wounds to which carbolic acid has been previously applied in order to check the prolonged action of the carbolic acid. Alcohol has also been employed as an antidote to the internal action of carbolic acid. A review of the chemical properties and relations of the two substances gives no indications of the possible nature of the assumed antagonism. It is obviously possible that the relationship may be either chemical or physical. The suggestive investigations of Sollmann, published a year ago, tended to indicate that physical factors were largely concerned and might possibly explain the facts entirely. It is possible, however, to test experimentally the hypothesis of a chemical antagonism, and such an investigation forms the subject of the present communication.

Yeasts are all more or less sensitive to the presence of alcohol in their culture media. For this reason fermentation ceases in many wines before the sugar contained in the grape-juice has been wholly converted into alcohol and carbon dioxide. Not only are the living yeast cells affected by higher concentrations of alcohol; the activity of the isolated ferment, the zymase, is also depressed by the presence of more than minimal amounts of alcohol. In this respect, however, yeasts vary. Through the kindness of Dr. Arthur Lachmann I have come into the possession of a wine yeast that ferments sugar in the presence of 15 per cent of alcohol. In a solution of 10 per cent of alcohol this yeast ferments sugar quite as actively as in the absence of alcohol. Since this yeast is very sensitive to the antiseptic action of carbolic acid, it presents a material for direct experimentation on the relations between alcohol and carbolic acid. In the ulti-

mate interpretation of the experimental results, the argument obviously assumes a complete analogy in this regard between the yeast cell and the animal cells. If alcohol be held to detoxicate carbolic acid in its action upon animal cells, we assume that it should diminish the toxic, that is, the antiseptic action upon yeast cells. If the carbolic acid were in any way chemically detoxicated by the alcohol, its antiseptic property ought to display a corresponding reduction. It is, of course, well known that plant and animal cells display widely varying reactions to chemical substances, dependent in many instances upon variations in oxidation—and reduction—ferments. In the systematic investigations of the reactions of chemical substances in plant and animal cells, any parallelism could in no wise be assumed. In the present instance, however, dealing with a substance like carbolic acid, possessing highly toxic action upon both plant and animal cells, it may be fairly assumed that the *modus operandi* of this toxic action (and *ipso facto* of its detoxication) is the same in both instances.

The experiment calls for a series of fermentation tests with varying concentrations of carbolic acid (from 1 : 1000 to 1 : 500,000) mixed with standard solutions containing 10 per cent of alcohol, 2 per cent of glucose, 0.5 per cent of peptone and the salts commonly added to the culture media employed with yeasts. The velocities of the fermentations, measured in terms of carbon dioxide, were compared under constant conditions of control with those displayed in exactly similar tests in the absence of carbolic acid. The experiments were repeated with variations in the temperature and in the contents of sugar, alcohol and yeast—always with the same results.

The results were entirely negative to the idea of a chemical detoxication of carbolic acid by alcohol. Alcohol does not reduce in the least the antiseptic action of carbolic acid. The yeast used in these experiments was sensitive to as high dilutions of carbolic acid as 1 : 100,000; at 1 : 10,000 fermentation was practically inhibited. This marked action of carbolic acid was in no wise retarded or inhibited by the presence of 10 per cent of alcohol. The presence in the media of soluble sulphates reduced appreciably the toxicity of the carbolic acid; but alcohol in no concentration had any such effect.



On the contrary, with high concentrations of alcohol and low concentrations of carbolic acid, the alcohol seemed to increase to some extent the toxicity of the carbolic acid. A 1 : 100,000 concentration of carbolic acid was more toxic in the presence of 10 per cent of alcohol than without it. This is in all probability an expression of the action of the alcohol upon the outer membrane of the yeast cells, rendering it more permeable to the entrance of the carbolic acid. The presence of as much sodium chloride as the yeast is known to tolerate alone, heightened the toxic action of the carbolic acid. This is, however, an expression of the law of partition; the presence of the sodium chloride renders the culture medium less of a solvent for carbolic acid, which has the effect of increasing the relative solubility of carbolic acid in the protoplasm of the cells. The presence of alcohol in the culture medium renders it a better solvent for carbolic acid, which would tend to reduce its relative solubility in the cell protoplasm, thus lowering its toxicity; since the contrary result in toxicity is observed, we are warranted in interpreting this as the effect of the alcohol upon the membrane of the yeast cells, whereby it is rendered more permeable to carbolic acid.

These experiments tend therefore to prove that there is no chemical detoxication of carbolic acid by ethyl alcohol, and that the effect observed in therapeutic practice must rest upon some physical basis. With this conclusion the more recent investigations of Sollmann are in full accord.



## THE BLOOD CLOT OF LIMULUS POLYPHEMUS.

By C. L. ALSBERG AND E. D. CLARK.

(From the United States Bureau of Fisheries Laboratory at Woods Hole, and the Department of Biological Chemistry of the Harvard Medical School.)

(Received for publication, August 22, 1908.)

As far as we are aware no analytical investigation of the composition of invertebrate blood cells has been published; and we know of no efforts to isolate the substances of which such cells are composed. The present research was begun as an attempt to undertake such studies upon favorable material. It was thought that *Limulus* would prove excellent for the purpose because of the ease with which considerable quantities of blood may be procured from this animal, and also because, as has been shown by L. Loeb,<sup>1</sup> there is but one kind of cell in the blood. However, we soon abandoned the attempt to study unchanged *Limulus* blood cells because of the difficulty of preventing large quantities of blood from clotting. Coagulation may be prevented by the methods of both Halliburton<sup>2</sup> and L. Loeb.<sup>3</sup> These methods necessitate the working up separately of small quantities of blood and involve more labor than we were able to give. In the light of our present knowledge such a study of unaltered cells seems worth this labor, and, it is hoped, will be attempted. In the present investigation only the clot was studied. Halliburton<sup>4</sup> believed that material from the plasma entered into the clot in addition to that derived from the cells. Howell,<sup>5</sup> on the

<sup>1</sup> *Folia Haematologica*, iv, May, 1907.

<sup>2</sup> *Journ. of Physiol.*, vi, pp. 300-335, 1885.

<sup>3</sup> L. Loeb: Ueber die Koagulation des Blutes einiger Arthropoden, *Beitr. z. chem. Physiol. u. Pathol.*, v, p. 192; Vergleichende Untersuchungen über die Thrombose, *Arch. f. path. Anat. u. Physiol.*, clxxxv, 1906; Studies on Cell Granules and Amoeboid Movements of the Blood Cells of *Limulus*, *Univ. of Pa. Med. Bull.*, May, 1905.

<sup>4</sup> *Loc. cit.*

<sup>5</sup> *Johns Hopkins University Circ.*, v, p. 4, 1885.

other hand, believed, upon purely morphological evidence, that the cells alone form the clot. Microscopical appearances, however, can not be regarded as capable of settling such a question as this. Experimental evidence is needed, and this has been furnished by L. Loeb<sup>1</sup> who, by showing that fibrinogen cannot be prepared from *Limulus* blood and that the plasma deprived of its cells cannot be made to clot, has established the fact that the clot of *Limulus* is formed of the agglutinated cells. We have not specially concerned ourselves with this question. We have made no attempt to prepare fibrinogen; but numerous incidental observations as well as our inability to obtain unmistakable fibrin from the clot bring our results in harmony with Loeb's finding that fibrinogen is absent.

We were also able to observe the apparent secondary coagulation described by Loeb.<sup>2</sup> We have not yet completed our investigation of the material involved in this phenomenon. Our studies, as far as they have at present proceeded, give no indication that a formation of fibrin takes place, and therefore support Loeb's interpretation of this phenomenon.

Our present paper deals merely with the chemical composition of the insoluble material of the clot. Following Loeb, we propose to call it cell-fibrin.

The clot is composed of tough elastic fibrous masses, which, when freed from adherent serum, are white or slightly yellowish. The clot itself does not reduce Fehling's solution, but does so powerfully after hydrolysis with mineral acids. The serum does not behave in this way. L. Loeb<sup>3</sup> made a simple chemical study of the clot, with which our findings are consistent. He found that the clot swells in alkalies but not in acids. It is coagulated by heat. After long boiling with 5 per cent potassium hydroxide he was unable to get a reduction of cupric oxide. This is all quite consistent with our findings that reduction takes place only after inversion. Loeb concludes that it is not mucin. This, as will appear, is also our own conclusion.

We prepared the insoluble protein of the clot, the cell-fibrin,

<sup>1</sup> *Beitr. z. chem. Physiol. u. Pathol.*, vi, p. 279; *Arch. f. path. Anat. u. Physiol.*, clxxiii, p. 48.

<sup>2</sup> *Beitr. z. chem. Physiol. u. Pathol.*, v, p. 194.

<sup>3</sup> *Arch. f. path. Anat. u. Physiol.*, clxxiii, p. 37.

as follows: The pieces of clot after thoroughly washing with 5 per cent sodium chloride were ground to a fine pulp in a porcelain ball-mill with 5 per cent sodium chloride. The salt solution was removed by straining through raw silk, and the extraction repeated. The material was then ground up repeatedly with distilled water. It was quite insoluble in distilled water, salt solutions and dilute acids; but slightly soluble in alkaline carbonates, and readily soluble only in caustic alkalies. It was, therefore, ground up in the mill with 2 per cent sodium hydroxide, filtered, and the clear filtrate precipitated by means of an excess of acetic acid. This precipitation was repeated. The acetic acid did not completely precipitate the protein. After removal of the acetic acid precipitate, a little more could be precipitated from the clear filtrate by hydrochloric acid. Whether this precipitate is identical with the acetic acid precipitate could not be decided for lack of material.

The cell-fibrin prepared in this way still gave a good reduction with Fehling's solution after hydrolysis with mineral acid. It also gave the Molisch-Udransky and the orcin reactions. We therefore assumed it to be a mucoid. On analysis, however, it proved to be poor in sulphur (0.62 per cent). In a mucoid containing chondroitin-sulphuric or glucothionic acid more sulphur is to be expected. Moreover the small sulphur content pointed to a very large molecular weight. Inasmuch as this protein does not exist preformed in the blood, but must be formed in the process of clotting, it is probably a decomposition product of a more complex substance of even greater molecular weight. These considerations led us to suspect the purity of our preparations and to endeavor to purify them further. After repeated solutions in dilute sodium hydroxide and precipitation with acetic acid, the power to reduce Fehling's solution, or to give the Molisch-Udransky and orcin reactions disappeared. The substance which is responsible for these reactions will form the subject of a future communication. The preparations were then washed salt-free, treated with alcohol and ether, and dried in the Schmiedeberg drying apparatus *in vacuo* over sulphuric acid at 70° C.

Preparation I gave the following analytical results:

0.2097 gm. of substance gave 26.8 cc. dry N at  $17^{\circ}$  and 768.5 mm.:  
N = 15.21 per cent.

0.2163 gm. of substance gave 27.4 cc. dry N at  $17^{\circ}$  and 768.5 mm.:  
N = 15.08 per cent.

0.2466 gm. of substance gave 0.1565 gm.  $H_2O$  : H = 7.10 per cent.

0.2129 gm. of substance gave 0.3771 gm.  $CO_2$  and 0.1336 gm.  $H_2O$  :  
C = 48.31 per cent; H = 7.02 per cent.

0.2288 gm. substance gave 0.4039 gm.  $CO_2$ ; C = 48.14 per cent.

0.4692 gm. substance fused with  $Na_2O_2$  according to the method of  
Folin, gave 0.0213 gm.  $BaSO_4$  : S = 0.61 per cent.

These figures do not represent the true percentage composition because unfortunately no ash determination was made and they can not be recalculated for the ash-free substance. It was not supposed that after the frequent reprecipitations and thorough washing appreciable amounts of ash could still be present. Such is, however, the case as an ash determination on Preparation II, which had been dissolved in sodium hydroxide and precipitated oftener than Preparation I showed.

0.2429 gm. of substance yielded 0.0107 gm. ash or 4.3 per cent. The ashing was done in platinum, and after the weight had been recorded the ash was boiled out with hydrochloric acid. Very little went into solution, the bulk of the ash consisting of silica. There was present also a little calcium but no magnesium, iron, copper or phosphorus. Whether the silica is present in organic combination with the protein or is merely an accidental contamination from the mill is a question which will be subjected to further investigation.

The analytical figures obtained for Preparation II checked fairly well with those obtained for No. I, though they were a little lower, pointing to a little greater ash-content for No. II. They are as follows:

0.1981 gm. substance gave 24.9 cc. dry N at  $18^{\circ}$  and 770 mm.: N =  
14.94 per cent.

0.1985 gm. substance gave 0.3495 gm.  $CO_2$  and 0.1229 gm.  $H_2O$ : C =  
48.02 per cent; H = 6.9 per cent.

Recalculated for the ash-free substance these figures become: C, 50.15;  
H, 7.1; N, 15.60 per cent.

In order to verify the sulphur determination a third Preparation was used which had been precipitated once more than Pre-

paration II. As the sulphur content was so low a great deal of material was used to minimize the errors of the method.

1.2666 gm. substance yielded 0.0516 gm.  $\text{BaSO}_4$ ; S = 0.55 per cent. This preparation contained 4.2 per cent of ash. The sulphur content of the ash-free substance then becomes 0.57 per cent.

These results were controlled by a determination in which the oxidation was carried out in silver by fusion with a mixture of sodium hydroxide and potassium nitrate. The result did not check up very well, but was quite a little lower than in either of the others.

To summarize, the substance has the following characteristics: It is soluble readily only in caustic alkalis, and precipitated from its solutions by acids, not being soluble in an excess. It gives the biuret reaction well; Millon's very faintly, if at all; Hopkins-Cole weakly but distinctly. Molisch's reaction and the orcin reaction are negative. Boiled with an alkaline lead solution it fails to give more than a faint trace of black color. Hence there is no evidence that it is in any way related to fibrin. It contains little or no tyrosin (negative Millon reaction) and is poorer in carbon, nitrogen and sulphur than fibrin, though richer in oxygen. It is of course possible that the low nitrogen and sulphur content is due to the treatment with alkali. To test this question we subjected some fibrin, prepared by defibrinating ox blood and washing, to exactly the same treatment. It was dissolved in alkali of the same strength in a ball-mill and the alkali allowed to act for the same length of time. It was precipitated with acetic acid and redissolved in alkali. At this point its behavior was quite different. The *Limulus* material went into resolution with difficulty, the fibrin with ease. The fibrin was reprecipitated and washed salt-free and analyzed. It contained 1.22 per cent of sulphur and 15.85 per cent of nitrogen (Kjeldahl). Evidently, therefore, if the *Limulus* material owes its low sulphur content to the treatment with alkali, it must be derived from some other material than ordinary mammalian fibrin. Whether the fibrin of invertebrates would behave in the same way on solution in alkali is, of course, unknown. We ought really to have used such a fibrin, but as nothing is known about it, it would have involved an independent research such

as we hope to undertake at another time. Finally, it must be borne in mind that we have no other guarantee than the fairly constant percentage composition that we are dealing with a pure substance.

Our substance is clearly an albuminoid and its low sulphur content as well as the negative or low tyrosin content point as clearly to a relationship with the glutin or elastin group. It differs from both in having a larger oxygen content, about 26 per cent. Elastin has a little less than 22 per cent; collagen nearly 25 per cent; and the collagen derivative, gelatin, about 25.25 per cent. As far as the oxygen content is concerned, our substance, therefore, is nearer to the glutin group. Its sulphur content, which is higher than that of elastin, also points to its belonging among the glutins. Its solubilities also fit in better with the glutins. It is not so insoluble as elastin. It is, to be sure, more soluble than collagen itself, and far less soluble than gelatin; but there is one member of the glutin group which has somewhat similar solubilities. This is the glutolin discovered by Faust<sup>1</sup> in the blood serum of the horse. This substance has about the same sulphur and hydrogen content, though about 1 per cent more carbon and 2 per cent more nitrogen. Only in its low nitrogen content does our substance differ very profoundly from glutolin. In spite of the fact that it contains 4 per cent less carbon than elastin, the C:N ratio of our substance (37.5:10) is almost the same as that of elastin and quite different from that of glutolin (34:10). In this respect our substance is near to fibrinoglobulin or fibrinogen, though there are no other signs of relationship, and many totally dissimilar properties (among others C:S ratio).

The C:N ratio of glutolin itself is, as Faust pointed out, nearer to that of the ordinary proteins than to that of the other members of the glutin group; and he, therefore, gave it an intermediary position between the other glutins and the ordinary proteins.<sup>2</sup> The glutins can be arranged in a series according to their C:N ratios beginning with gelatin, with the highest nitrogen content, and ending with glutolin, with the lowest nitrogen content;

<sup>1</sup> *Arch. f. exp. Path u. Pharm.*, xli, p. 309ff.

<sup>2</sup> *Loc. cit.*, p. 321.



collagen and conchiolin occupying an intermediary position.<sup>1</sup> Our substance would be still more extreme even than glutolin, having both a lower nitrogen content and a higher oxygen content. In this respect it is nearer the ordinary proteins. It, nevertheless, seems to belong rather to the glutin (or elastin) group, as its insolubility, its low sulphur content, its negative Millon reaction, clearly indicate. Definitely to settle its position will, however, require a study of its component amino-acids, a task that will be undertaken with more material in the future. It is hoped that other properties not hitherto studied because of lack of material will then be investigated.

If we are in fact dealing with a member of the glutin group, it is not many times before that such a substance has been isolated from an invertebrate and characterized chemically. According to v. Furth<sup>2</sup> collagen has never been convincingly demonstrated in invertebrates. It is interesting to note that an animal which, like *Limulus*, has the matrix of its skeleton composed of chitin instead of collagen, perhaps contains a glutin substance. It will be interesting to investigate, as we propose to do, whether the tendons, etc., of this animal contain a glutin, and if so, whether it resembles the cell-fibrin we have described.

It is, moreover, interesting that the cells of the blood of invertebrates have many points in common with the platelets of higher forms.<sup>3</sup> Whether the similarity extends to the chemical composition is a question that demands investigation. Perhaps agglutination thrombi are composed of a material like *Limulus* cell-fibrin. It is not beyond the range of possibilities, judging by the points of resemblance between *Limulus* cell-fibrin and Faust's glutolin, that the latter may be derived from the platelets. We, therefore, propose to study the platelets from this point of view.

<sup>1</sup> Cf. Faust: *Loc. cit.*, p. 319.

<sup>2</sup> *Chemische Physiologie der niederen Tiere.*

<sup>3</sup> Deckhuyzen: *Anat. Anz.*, xix, 1906; L. Loeb: *Beitr. z. chem. Physiol. u. Pathol.*, v, p. 197; *Folia haematologica*, iv, May, 1907.



## THE EFFECT OF DIET ON THE MALTOSÉ-SPLITTING POWER OF THE SALIVA.

By CHARLES HUGH NEILSON AND M. H. SCHEELE.

(From the Physiological Department of St. Louis University.)

(Received for publication, July 31, 1908.)

The most important enzyme of the saliva is ptyalin. Its action according to the best authorities is the splitting of starch into maltose. Some investigators say it also acts on maltose, splitting it into dextrose. Not much stress is laid on the supposed presence of a second enzyme in the saliva, namely, maltase. In several of the best text-books on physiology, however, it is mentioned as being present in the saliva.

It occurred to us that the maltase in the saliva might be increased on a carbohydrate diet and decreased on a protein diet. Neilson and Lewis<sup>1</sup> have shown an increase in the amylolytic power of human saliva on a carbohydrate diet and a decrease on a protein diet. The object of this paper is to determine whether a similar change may be found in the action of saliva on splitting maltose.

The "adaptation" of the digestive glands to the character of the food has received considerable attention in recent years. Wassilief<sup>2</sup> and Lintwarew<sup>3</sup> have shown that in dogs the quality of the pancreatic juice as well as the quantity of the enzymes is dependent on the food of the animal. Ellinger and Cohn<sup>4</sup> have shown that the human pancreatic juice is affected by diet as Walter<sup>5</sup> has shown for the dog. Weinland<sup>6</sup> has shown that the pancreatic juice of the dog has lactase present on a diet con-

<sup>1</sup> Neilson and Lewis: *This Journal*, iv, p. 501, 1908.

<sup>2</sup> Wassilief: *Arch. des biol., St. Petersburg*, ii, p. 219, 1893.

<sup>3</sup> Lintwarew: *Biochem. Centralbl.*, i, p. 201, 1903.

<sup>4</sup> Ellinger and Cohn: *Zeitschr. f. physiol. Chem.*, xlv, p. 201, 1908.

<sup>5</sup> Walter: *Ibid.*, vii, p. 1, 1899.

<sup>6</sup> Weinland: *Zeitschr. f. Biol.*, xxxviii, pp. 16 and 607, 1899; xl, p. 386, 1900.

taining an excess of lactose. Neilson and Terry<sup>1</sup> have shown that the amylolytic action of dog's saliva is increased on a carbohydrate diet.

*Method.* The subjects for these experiments were students. No change was made in their manner of living other than the change in diet. This diet consisted of two kinds; one an excess of carbohydrate food; the other an excess of protein food. Coffee, tea, milk, or beer were allowed if the subjects were accustomed to use them. Since the taking away of liquids might increase the concentration of the saliva it was thought best to allow the usual liquids.

The saliva was first tested for three consecutive days while the subjects were on a mixed diet. In testing this saliva the following experiments were done:

A definite quantity of saliva (25 cc.) was collected. The amylolytic power of the saliva was first tested. Two and one-half cubic centimeters of saliva were diluted to 100 cc. with distilled water. Ten cubic centimeters of this diluted saliva were placed in a 250 cc. Erlenmeyer flask containing 75 cc. of 2 per cent arrow root starch and 15 cc. of water. The total contents of the flask were warmed to 37.5° C., the temperature of the incubator. The flasks were thoroughly shaken, placed in the incubator and left for 20 minutes. At the end of this time the contents were boiled, cooled to room temperature and the amount of maltose produced determined by Haines' titration method. A control was always made with boiled saliva.

The experiments to show the action of the maltase in the saliva were made as follows:

In a 250 cc. Erlenmeyer flask 1 gram of maltose in 45 cc. of distilled water was placed. The contents were warmed to 37.5° C. To one flask 5 cc. of undiluted saliva were added and the contents of the flask at once boiled. To the other flask 5 cc. of undiluted unboiled saliva were added. These flasks were placed in the incubator and left for one hour. At the end of this time the contents were boiled and made up to 100 cc. with distilled water. This dilution made a solution of 1 per cent maltose. The reducing power was determined by the Haines titration

<sup>1</sup> Neilson and Terry: *Amer. Journ. of Physiol.*, xv, p. 406, 1906.

method. The result from the flask with the boiled saliva was for a control. The result from the flask with the unboiled saliva showed the action of the maltase in the saliva by the increase in reducing power over the control. Maltose has approximately one-third less reducing power than dextrose. If maltase were present we would expect to find the reducing power of the solution with unboiled saliva to be greater than the boiled saliva. This increase in the reducing power of the solution with the unboiled saliva is due to the action of the maltase in the saliva splitting the maltose into dextrose. This increase was found in every case without exception.

A polarimeter determination was also made with the contents of each flask. The solution with the unboiled saliva always showed a smaller rotation than the solution with the boiled saliva. This result one would expect as the power of rotation of dextrose is much less than that of maltose. In many of the experiments it was necessary to filter through animal charcoal to clear the solution before the polarimeter readings were made.

The amylolytic power of the saliva of the subjects on a mixed diet and the power of this saliva in splitting maltose were used as controls for the saliva when the subjects were placed on a carbohydrate or protein diet. Twenty-four hours after the protein or carbohydrate diet was started, the amylolytic power of the saliva and its maltose-splitting power was determined.

The amylolytic power of the saliva was estimated in order to determine whether the change in maltose-splitting power was parallel with the change in the amylolytic power of the saliva from the subjects on the different diets.

In Table 1 the increase of the maltose-splitting power of the saliva from those on a carbohydrate diet and the decrease on a protein diet is seen. This change is calculated in per cent using as a basis the maltose-splitting power of the boiled saliva. Of course, the boiled saliva had no splitting power and its reducing power was that of pure maltose as none of the maltose had been split into dextrose. The reducing power of the boiled saliva was considered as 100 per cent. We now have the maltose-splitting power of the saliva from the subjects with stated diet. To show the effect of this diet these results are compared with the maltose-splitting power of the saliva from a mixed diet.

TABLE 1.  
EXPERIMENT 1.

DIET.	Day of diet.	1	2	3
		1 gram maltose, 45 cc. distilled water, 5 cc. boiled saliva.  Reducing power considered 100 per cent.	1 gram maltose, 45 cc. distilled water, 5 cc. unboiled saliva.  Change in reducing power.	75 cc. 2 per cent starch paste, 15 cc. distilled water, 10 cc. dilute saliva.  Amylolytic power of saliva.
		<i>per cent.</i>	<i>per cent.</i>	<i>mg. maltose.</i>
Mixed diet. Results average for three days.....		100	+23	121
Protein diet.....	1	100	+5	105
".....	2	100	+3	103
".....	4	100	+3	101
Carbohydrate diet.....	1	100	+12	111
".....	2	100	+38	180
".....	3	100	+39	180
Protein diet.....	1	100	+18	145
".....	2	100	+8	112

EXPERIMENT 2

DIET.	Day of diet.	1	2	3
		100	+18	110
Mixed diet. Results average for three days.....		100	+23	128
Carbohydrate diet.....	1	100	+33	151
".....	2	100	+35	160
".....	4	100	+16	110
Protein diet.....	1	100	+0.5	50
".....	2	100	+6	85
".....	3	100	+36	140
Carbohydrate diet.....	1	100	+35	145
".....	2	100		

In every experiment without exception a carbohydrate diet increased the maltose-splitting power of the saliva and a protein diet decreased it. This change is parallel to the change in the amylolytic power of the saliva on the same diet.

TABLE 2.  
EFFECT OF VARIATION IN CONCENTRATION OF MALTOSE.

*The time and amount of saliva are constant.*

SOLUTION.	5 grams maltose.	1 gram maltose.	1.5 grams maltose.	2 grams maltose.	4 grams maltose.
	per cent.	per cent.	per cent.	per cent.	per cent.
The reducing power of each amount of maltose plus					
45 cc. water and	100	100	100	100	100
5 cc. boiled saliva					
45 cc. distilled water	126	132	130	120	116
5 cc. unboiled saliva					

EFFECT OF VARIATION IN AMOUNT OF SALIVA.

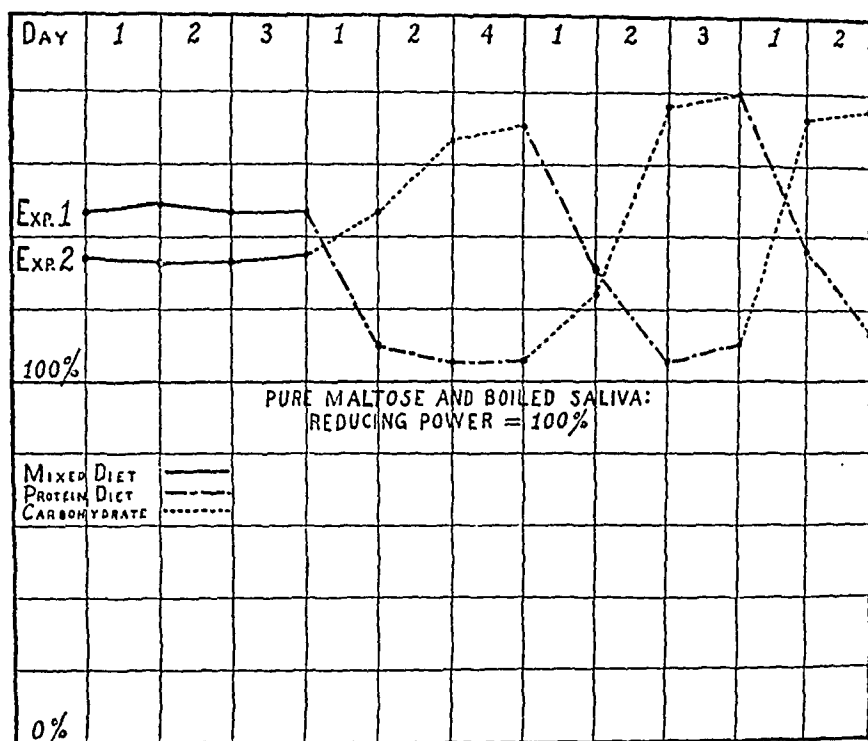
*The time and amount of maltose are constant.*

	2.5 cc. saliva.	5 cc. saliva.	10 cc. saliva.
	per cent.	per cent.	per cent.
The reducing power of each solution containing			
1 gram of maltose and the different amounts of boiled saliva plus enough water to make 50 cc . . . . .	100	100	100
1 gram. of maltose plus the different amounts of unboiled saliva plus enough water to make 50 cc . . . . .	116	133	132

EFFECT OF VARIATION IN TIME.

*The amount of maltose and saliva are constant.*

SOLUTION.	REDUCING POWER AND TIME.		
	$\frac{1}{2}$ hour.	1 hour.	$1\frac{1}{2}$ hour.
	per cent.	per cent.	per cent.
1 gram maltose	100	100	100
45 cc. water			
5 cc. boiled saliva (control)			
1 gram maltose	116	130	134
45 cc. distilled water			
5 cc. unboiled saliva			



In all the experiments polarimeter determinations were made and they always agreed with the results obtained by the reduction method. The polarimeter readings are not given as they would make the tabulated results too complex.

In Table 2 the effect of variation in time, amount of maltose and the amount of saliva is seen. These experiments show that in the higher concentrations of maltose, for instance, a 4 per cent and 8 per cent solution (2 grams and 4 grams in 50 cc. of solution) the maltose-splitting power of the saliva is lessened. The optimum concentration is 2 to 3 per cent maltose. The amount of splitting is not proportional to the amount of saliva used in the experiments. It is proportional for 2.5 cc. and 5 cc. but not when 10 cc. were used. The amount of splitting is not directly proportional to the length of time of the experiment.

In diagram above a curve to show the change in the maltose splitting power of the saliva on different diets is given.



We see from these experiments that a change of diet produces a change in the maltose-splitting power of the saliva. The change is parallel to the change in the amylolytic power of the saliva on the same diets. As the amylolytic power of the saliva increases on a carbohydrate diet, the maltose-splitting power increases in approximately the same ratio. On a protein diet the amylolytic power and the maltose-splitting power decrease in the same ratio. These experiments seem to add further proof in favor of adaptation to diet.

If the action of ptyalin and maltase be specific, then the explanation lies in the increase of the amount of maltase or the secretion of a maltase with greater splitting power. The increase of the concentration of the saliva on a carbohydrate diet with a relative increase in the amount of maltase would also offer an explanation.

If the ptyalin has both an amylolytic and maltose-splitting power the increase in the quantity or action of the ptyalin would explain these results. No attempts were made to determine whether the result is due to the action of ptyalin or to the action of the maltase.



# SOME NOTES ON THE CHEMICAL COMPOSITION AND TOXICITY OF *IBERVILLEA SONORAE*.

(PLATES IV AND V)

By JULIA T. EMERSON AND WILLIAM H. WELKER.<sup>1</sup>

(From the Laboratories of the New York Botanical Garden and the Laboratory of Biological Chemistry of Columbia University, at the College of Physicians and Surgeons, New York.)

(Received for publication, September 2, 1908.)

	Page
I. Introduction.....	339
II. Historical.....	340
III. General description of the species <i>Sonora</i> .....	340
IV. General quantitative composition.....	341
V. General qualitative composition.....	342
VI. Toxicological notes.....	345
VII. General conclusions.....	350

## I. INTRODUCTION.

Among the many plants about which we know little or nothing chemically is *Ibervillea Sonora*. Some of the largest tubers of this species were collected by Dr. D. T. MacDougal in Sonora, N. M., in 1902. At the suggestion of Dr. MacDougal, and under the direction and with the assistance of Dr. William J. Gies, this study of the stem<sup>2</sup> ("root") was undertaken. An effort was made to ascertain its general chemical composition. We also endeavored to determine the nature of the substance or substances in it which account for the poisonous qualities attributed to it by the inhabitants of the regions in which it is found.

<sup>1</sup> The work on chemical composition was done by Miss Emerson at the New York Botanical Garden. The experiments on the toxicity of the root were carried out by Mr. Welker in the Laboratory of Biological Chemistry of Columbia University, at the College of Physicians and Surgeons, New York.

<sup>2</sup> Knox: *Bulletin of the Torrey Botanical Club*, xxxiv, p. 329, 1907. See the second footnote on p. 341

## II. HISTORICAL.

(1) A genus under the name of *Sicydium* was described by Schlechtendal in 1832 (*Linnæa*, vii, p. 388), based upon *Sicydium Schiedeianum*, a Mexican plant.

(2) Gray described in 1850 *Sicydium Lindheimeri* (*Bost. Jour. Nat. Hist.*, vi, p. 194) from Texas.

(3) *Sicydium Lindheimeri* was made the type of a new genus, *Maximowiczia*, by Cogniaux, in 1881 (*D. C. Monog. Phan.*, iii, p. 726).

(4) *Maximowiczia Sonoræ* was described by Sereno Watson in 1889 (*Proc. Amer. Acad. Arts and Sci.*, xxiv, p. 51).

(5) As the name *Maximowiczia* had been used before 1881 for another genus (by Ruprecht in 1859, for a genus of Magnoliaceæ), Greene (*Erythea*, iii, p. 75, 1895) substituted for the cucurbitaceous genus the name *Ibervillea*, and *Maximowiczia Sonoræ* thus became *Ibervillea Sonoræ*.

## III. GENERAL DESCRIPTION OF THE SPECIES SONORÆ.

*Ibervillea* belongs to the Cucurbitaceæ or Gourd family, and has been found in Mexico, New Mexico, Texas, and California. As Watson's (4) is the only full description of the species *Sonoræ*, it is quoted below.

Climbing, glabrous throughout, the large root (stem) projecting above ground; leaves four inches broad or less, twice three-cleft nearly to base, with broad sinuses, the lobes coarsely sinuate-toothed; male flowers racemose, short-pedicellate, the calyx-tube cylindrical, 3 lines long; petals pubescent, villous within, bifid; fertile flowers on peduncles 2 to 3 lines long; ovary ovate, long-attenuate above; fruit ovate, abruptly stout beaked, amber colored  $1\frac{1}{2}$  to  $1\frac{1}{4}$  inch long, smooth with a thick fleshy rind; placentæ many (about 14) seeded; seeds covered with a red pulp, compressed, rough coated excepting the smooth margin, obovate or oblong-obovate with a broad base, 3 lines long or more.

This species much resembles *M. Lindheimeri*, but the leaves are more dissected and the ovary and fruit more attenuate above, and the seeds though decidedly turgid in the young fruit, become compressed and are peculiar in their generally very rugose-tuberculate surface. The green fruit is described as having about ten longitudinal rows of white dots.

The upper side of the swollen underground portion where it is exposed to the light is of a green-gray color owing to chlorophyll just beneath the periderm, while the upper side is thicker, brown

and without the green. The substance of the "root" is much the same throughout; firm, fibrous near the stem or a bud, more difficult to cut and tougher than a potato, and of a light yellow color. Its general shape is round, like a gourd, with a neck leading up to the shoots and several thick roots extending downwards, and it may become as large as two feet in diameter and weigh many pounds. There is a characteristic odor somewhat like that of an earthy potato, and after working with fresh material some time the nose and throat feel dry and irritated, while a bitter tasting substance remains on the fingers, even after several washings.

Figs. A and B show the organographic characters of the species, both at the adult stage in its native habitat, and as a seedling from four to five years old. The large projecting root of *Ibervillea* in the specimens in the greenhouses of the Botanical Garden reaches a diameter of from 25 to 30 cm. In the desert this enormous tuberous growth lying in the dry sand looks like a gray, dust-covered boulder. Frequently irregularities of shape give it still more the effect of stone, and it is only when the cortex is flecked off that one discovers the healthy green color beneath the superficial layer. From the tubers arise yearly long flexible liana-like shoots which reach a length of three or more meters. The shoots are round, smooth and green above, brown-gray and gray-spotted or streaked below. The flowers are dioecious, the tendrils branched, and the leaves bright-green and twice three-cleft as is so frequently the case throughout the family. The fruit is said to be "amber colored" and  $1\frac{1}{2}$  to  $1\frac{3}{4}$  inch long; none has ripened in the greenhouse, as the flowers there are staminate only. The plant is able to persist in its arid habitat with remarkable vitality. In fact, so provident is it of water and nutritive substances that one in the museum case at the Garden which has been lying on a board since 1902 is, in 1908, still sending up yearly shoots bearing leaves and tendrils. Every fall the shoots die back and sprout again early the next spring.<sup>1</sup>

#### IV. GENERAL QUANTITATIVE COMPOSITION.

Water, solids, organic and inorganic matter in the stem were determined by the usual methods. After removal of the bark, fresh portions of the stem<sup>2</sup> were quickly cut into small pieces and

<sup>1</sup> Knox: *Loc. cit.* This paper gives the results of a thorough anatomical study of the stem ("root") of *Ibervillea Sonora*.

<sup>2</sup> This tuber is ordinarily referred to as a root \* \* \* The picture of the old plant (Fig. A) with its shoots rising from the tuber, shows the gradual enlargement of the stem, though the appearance of the seedling

weighed in porcelain crucibles as rapidly as possible. Each sample was dried to constant weight in an air bath at 100° C., in which process all water was removed. The weight of the dry solid matter was taken and, after complete incineration at a low temperature, the weight of the ash was also noted. The following weights and percentages were recorded in this connection:

## RECORDED ANALYTIC DATA.

Tuber Sample.	Fresh substance. grams.	Dry substance. grams.	Ash. gram.
1.....	12.0060	1.6690	0.1710
2.....	15.4403	1.9223	0.2203
3.....	19.3512	2.3222	0.3822
4.....	14.9534	1.9834	0.2144
Average .....	15.4377	1.9742	0.2219

## GENERAL PERCENTAGE COMPOSITION.

Tuber Sample.	Water.	Solids.	Organic matter.	Inorganic matter.
1.....	86.10	13.90	12.49	1.41
2.....	87.56	12.44	11.02	1.42
3.....	88.00	12.00	10.60	1.40
4.....	86.74	13.26	11.82	1.44
Average .....	87.10	12.90	11.48	1.42

## V. GENERAL QUALITATIVE COMPOSITION.

In an endeavor to ascertain the character of the more conspicuous constituents of the tuber, grouped under the general term "solids," various extracts were made and tested. Between the arrival of the tubers from Sonora and the time when they were used for this study (almost a year), they remained in the laboratory on saucers, without earth or water, and seemed to keep perfectly, one even putting out several shoots during the summer.<sup>1</sup>

*Preparation of material.* In the preparation of the tuber for these tests, dirt and dust were brushed off and the tuber was

(Fig. B) would indicate that the swollen portion includes root, hypocotyl and stem. \* \* \* The formation is stem and at least half of the swollen portion may claim that distinction.—Knox, *loc. cit.*, p. 340.

<sup>1</sup> The quantitative data recorded above were obtained from tubers in the same general condition.

washed. Sometimes the bark was removed; at other times the entire root, after removal of injured or abnormal portions, was finely divided in a meat chopper. Portions of the chopped tuber were at once put in bottles with one of the solvents named below, a few drops of toluol or chloroform being added as preservatives. After standing generally a week, the extract was filtered and this filtrate used in the experiments.

Extracts were made with the following solvents: Water, 95 per cent alcohol, ether, glycerol, 10 per cent sodium chlorid, or acidified water (very dilute hydrochloric acid). The aqueous and saline extracts were slightly acid in reaction (litmus).

The common qualitative reactions on these extracts made it evident that the root contains representatives of the following groups of substances: proteins, carbohydrates, fats, cholesterins, lecithins, basic substances, salts of organic acids, and inorganic salts.

*Proteins.* The simple proteins present in the tuber were of the coagulable type only. Proteose and peptone could not be detected in any of the extracts after removal of the coagulable proteins. Nucleoprotein was present in small proportions in the extracts and could be precipitated by acid. On decomposition in hot acid the protein yielded purin bases, simple protein and phosphate.

It was hoped by finding the temperature at which coagulations resulted in the extracts, to gain some idea of the number and types of the coagulable proteins present. The results were not very definite, however. Ten per cent salt extract, for example, showed turbidity at the low point of 25° C. The temperature was gradually raised and at 61° C. a fine flocculent precipitate appeared. On filtering, a clear yellow filtrate was obtained. On reheating, initial turbidity became apparent above the temperature of former turbidity but below that of former flocculation; in this way six to eight flocculations were obtained at temperatures ranging between 25° C. and 80° C.

On adding to an aqueous or saline extract an equal volume of saturated ammonium sulfate solution, a fairly bulky precipitate of "globulin" was obtained. The half saturated filtrate gave an abundant yield of albumin when ammonium sulfate was added to complete saturation. Saturation of the saline extract with

magnesium sulfate precipitated globulin. Solutions of both the globulin and the albumin precipitates obtained in this way gave no more satisfactory differential results with the coagulation method than the original extracts. The number of globulins and albumins present was not, therefore, ascertained. It appeared probable that these proteins coagulated through ranges of degrees of temperature that overlapped each other, so to speak, thus making differentiation by this process impossible, or else that other substances were precipitated in such ways as to interfere in the manner indicated.

The precipitates obtained at some of the lower temperatures may have consisted largely of earthy phosphate, for they were not appreciably soluble in 10 per cent sodium chlorid solution or in 0.5 per cent sodium carbonate solution, but readily dissolved in 0.2 per cent hydrochloric acid.

The precipitates obtained at the higher temperatures, on the other hand, were true coagula, for they were practically insoluble in all of the three reagents named.

*Carbohydrates.* The carbohydrates present in the tuber were chiefly woody fiber and small amounts of reducing sugar and starch. The nature of the small amount of sugar was not determined.

*Fat, cholesterin and lecithin* were extracted and separated by Zuelzer's ether-acetone method.<sup>1</sup> No attempt was made to ascertain the characters of either the fatty matter or the lecithin. Their quantities appeared to be relatively slight.

*Saline matters*, especially chlorid, sulfate and phosphate, sodium, potassium, calcium, magnesium and iron could be readily detected. The ash contained carbonate, most of which was formed by the combustion of the organic matter. Salts of organic acids were frequently indicated, in the various reactions tried, but no attempt was made to ascertain the characters of the organic radicals. Tri-basic calcium phosphate was precipitated in relative abundance from aqueous and saline extracts on warming. Earthy phosphate was subsequently found to occur in the root, and in such extracts.

*Enzymes* were looked for but were not detected. Proteolytic,

<sup>1</sup> Zuelzer: *Zeitschr. für physiol. Chem.*, xxvii, p. 255, 1899.



diastatic and oxidative enzymes appeared to be absent. It is possible that the conditions of the tests were not favorable to digestive results, although ordinary methods were used.

*Basic substances.* A few tests for alkaloids, with the so-called alkaloidal reagents, in extracts free from coagulable protein, gave indications of the presence of basic substances (see p. 346).

*Chlorophyll* was conspicuous in the subcortical tissue.

## VI. TOXICOLOGICAL NOTES.

In Mexico where *Ibervillea Sonoræ* grows in abundance, the natives consider it poisonous. Watson<sup>1</sup> has stated that "a decoction of the root is used as a cathartic."

*Preliminary toxicological experiments on frogs.* Several preliminary tests of the toxicity of the tuber were made on frogs, by injecting under the skin or into the stomachs small volumes of concentrated aqueous solutions of dry residues from alcohol or ether extracts, with the following results:

(a) Residue from *alcohol* extracts. First frog: Introduction *per os*. No visible effects. Second frog: Injection subcutaneously. Spasms occurred in a few minutes; later there was retching. Death ensued in three hours.

(b) Residue from *ether* extracts. Third frog: Introduction *per os*, about 3 p.m. There were signs of nausea during the afternoon. Death occurred during the night. Fourth frog: Injection subcutaneously. Convulsive movements preceded death in 2½ hours.

The results of these tests led us to look for alkaloids. For the preliminary isolation of any of the latter that might have been present in the tuber we proceeded as is indicated below:

*Tests for alkaloids.* The Stas-Otto method was employed for the preparation of the extracts to be tested. The interior portions of two tubers were finely minced and subjected to extraction separately. Two alcoholic extracts were made of each. The residues from each yellow alcoholic extract, which were obtained by evaporation *in vacuo* at 40° C., were treated with absolute alcohol, filtered and again evaporated to dryness. The residues from the absolute alcohol were treated with water, the filtered

<sup>1</sup> See p. 340.

solutions rendered alkaline with sodium carbonate<sup>1</sup> and any alkaloids extracted with ether. Each residue was treated thrice with fresh ether. The ether extracts were allowed to evaporate to dryness spontaneously. The resultant dry residues, which were yellowish in color and very bitter, were used in the tests referred to below.<sup>2</sup>

*Chemical tests.* Samples of the various residues obtained by the above described method were dissolved in dilute hydrochloric acid or alcohol and tested with common reagents for alkaloids. White, amorphous precipitates were obtained with potassium hydroxid, phosphomolybdic acid, phosphotungstic acid and tannic acid.

Precipitates failed to form with solutions of picric acid, platinic chlorid, iodine in potassium iodid solution, bromine in hydrobromic acid solution, potassium sulphocyanid, potassium chromate, potassium dichromate, potassium iodid, mercuric chlorid, potassium ferrocyanid, potassium ferricyanid.<sup>3</sup>

The material in the extracts gave color reactions with the following reagents: Sulfuric acid, Erdmann's reagent, sulphovanadic acid, and Fröhde's reagent. None of these results was characteristic of any particular alkaloid, however, nor were mixtures of alkaloids suggested.

The reaction with sulfuric acid and potassium permanganate in its first stages resembled the coloration obtained with strychnine in the oxidation test, but it failed to exhibit the successive changes of coloration characteristic of the alkaloid in that reaction. The color was not given with any other oxidizing reagents in sulfuric acid. According to Wormley, this reaction, if potassium permanganate is employed as the oxidizing agent, is given

<sup>1</sup> The yellow color changed to orange.

<sup>2</sup> While we were removing residue from one of the evaporation dishes, an associate who was fully ten feet away and who knew nothing about the peculiar qualities of the residue, expressed surprise at a bitter taste he suddenly experienced. The very minute amount of dust that was formed as we carefully scraped the residue from the dish accounted for the experience referred to, which gives a good idea of the extreme bitterness of the material.

<sup>3</sup> Precipitates formed by reaction between the solvent and the reagent are here ignored.

by aqueous extracts of certain plants and even by shreds of filter paper.<sup>1</sup>

*Physiological tests.* Toxicity of the residues obtained by the method described above was determined by dosing a 2.7 kilo female pup as follows:

*Administration per os: 1st day.* The dog was given in one meal daily an ordinary quantity of food consisting of meat, cracker meal, lard, bone-ash and water. The weight of the residue given the first day was 0.0523 gram. It was placed inside of a ball of meat and in this condition swallowed without mastication. Except very slight diarrhea during the following night, no effects were observed.

*2d day.* On the second day of the experiment 0.1046 gram of residue was administered in the food as before. Slight diarrhea, about nine hours after feeding, was the only apparent effect of the material.

*3d day.* On the third day the animal was apparently perfectly normal, and accordingly a study of the effects of *subcutaneous* injections of the material was begun on the following day.

*Subcutaneous injection: 4th day.* The animal appeared to be normal in all respects. About 0.05 gm. of residue was dissolved in 3 to 4 cc. of alcohol, and injected on the right side. No general effects whatever were observed during the following twenty-four hours.

*5th day.* On this day about 0.1 gram of residue was injected in 3 to 4 cc. of alcoholic solution. No symptoms of any toxic action were observed during ten hours following the injection. A small abscess formed at the point of injection; otherwise the animal apparently remained normal during the next two days, when observation was discontinued.

These tests failed to show the presence in the extracted material of any known alkaloid or alkaloids. The physiological tests indicated further that, if any alkaloid was present, its proportionate content was too slight to enable it to exhibit distinct or characteristic effects.

*Tests of the effects of hashed tuber given by mouth to dogs.* The results of the foregoing toxicological tests suggested either that our extracts did not contain the toxic principle of the tuber or that the reputed poisonous action of the tuber is due to some compound other than an alkaloid. To decide this question, a number of special experiments on toxicity were conducted.

Two tubers were rapidly peeled, sectioned and hashed. The interior of one of them was found to be full of small brown masses. The other

---

<sup>1</sup> Wormley: *Micro-chemistry of Poisons*, 2d ed., p. 589, 1885; Sedgwick: *Amer. Chem. Journ.*, p. 369, 1879.

tuber was apparently normal in all respects. The mass of each tuber was kept separate on the assumption that possibly the abnormal specimen would show different toxic results from those of the normal tuber; also with the idea that perhaps the toxicity ascribed to *Ibervillea Sonorae* in general was due to special poisonous principles in such abnormal tubers and that particular effort should be made to determine this possibility. The rinds were also hashed. Each product was bottled and placed in a cold room which was kept constantly at a temperature of about  $-5^{\circ}\text{C}$ .

The following quotations from our records of this series of experiments will suffice to show the results obtained.

*Sixth experiment with normal tuber.* Weight of dog, 25.7 kilos.<sup>1</sup>

*1st day.* Sixteen grams of tuber were administered in balls of meat at 11 a.m. In the afternoon a feverish and restless condition was observed. This was very much less marked during the evening. Pronounced diarrhea occurred between midnight and 8 a.m. *2d day.* By 10 a.m. the condition of the dog was apparently very close to normal. Twenty-four grams of tuber were given in meat at 10.30 a.m. The feverish condition reappeared unmistakably at 1.45 p.m. Vomiting occurred at intervals between 5 and 9.45 p.m. No defecation occurred during the day, at the end of which the dog was apparently normal. *3d day.* Thirty-two grams of tuber were administered with the regular diet at 10.50 a.m. At 12.30 p.m. the animal became feverish and uncomfortable. Three hours later very marked diarrhea occurred. About 6 p.m. a considerable portion of the food given in the morning, including a fairly large proportion of meat, was vomited. Between 7 and 8 p.m. marked diarrhea occurred. From this time until the next morning, recovery was rapid. The dog was apparently normal at the end of that period. *4th day.* The dose of tuber was again increased and 40 grams were given with the usual diet between 11 a.m. and 1 p.m. At 1.40 p.m. the first signs of sickness appeared. Between 4.15 p.m. and 5.20 p.m. very marked diarrhea occurred. This was followed by rapid recovery and the dog was nearly normal four hours later. *5th day.* The animal refused to eat the meat balls containing the tuber and so was allowed to fast until the sixth day. *6th day.* The dog again refused the meat balls. They were given forcibly. He received 50 grams of tuber at noon. The feeding was followed by a five-hour period of feverishness and restlessness. This was succeeded by very marked diarrhea. By midnight the dog had practically recovered. He remained well. The same animal was used in the seventh experiment.

*Seventh experiment; with abnormal, i. e., "spotted" tuber, and with the rind.* About a month and a half after the completion of the preceding experiment, the dog of that experiment, which showed no subsequent ill effects of the treatment described above, was subjected to dosage with the abnormal tuber. Diet and method of dosage were the same as in the sixth experiment.

---

<sup>1</sup> Daily diet—hashed meat, cracker meal, lard and water, with bone-ash. A preliminary period of five days established normal conditions.

A. *1st day.* Five grams of "spotted" tuber were given without effect. The feces passed this day were hard and dry. *2d day.* Ten grams of tuber were given. There were no apparent effects. *3d day.* Twenty grams of tuber were administered in gelatin capsules. This dose also failed to produce any toxic symptoms. The feces remained hard and dry. *4th day.* Forty grams of tuber were given in capsules as before. Apparently no effects were produced. *5th day.* Fifty grams of tuber were given at noon in capsules as before. Slight diarrhea occurred during the afternoon and evening, while marked diarrhea ensued between midnight and 8 a.m. The dog was apparently normal at 9 a.m. *6th day.* The regular diet was fed without the tuber.

B. *7th day.* Twenty-five grams of rind were given in gelatin capsules with the regular diet. Very marked diarrhea occurred about six hours later. The dog seemed normal on the following morning. *8th, 9th and 10th days.* The dog was fed the usual diet without any rind. *11th day.* Twenty-five grams of rind were given in capsules without producing any apparent effect. *12th day.* Twenty-five grams of rind were given. Three hours later marked diarrhea ensued. This was followed by the vomiting of undigested meat. *13th day.* The animal fasted. *14th day.* A small meal was fed in the afternoon. *15th day.* Twenty-five grams of rind were given in capsules with the regular diet at 10 a.m. Diarrhea occurred between 8 and 8:45 p.m. By the following morning the dog had apparently recovered.

These experiments and the remaining ones of the series made it evident that the toxicity and even the cathartic effects of *Ibervillea Sonoræ*, as measured by results on our dogs, have perhaps been overrated. In some instances, comparatively large quantities of the hashed tuber or the rind were entirely devoid of obvious effects. The "spotted" tuber was no more (but perhaps less) toxic than the normal tuber. These results were also in accord with our previous conclusion that there is little if any alkaloid present in the tuber.

Although the toxic and cathartic effects were relatively slight, it was thought that possibly oxalic acid was present in sufficient proportion to share in producing the effects noted. Analysis of a fresh portion of tuber made it evident, however, that the proportion of oxalic acid in the tuber was very slight.

It also seemed possible that magnesium salts might have participated in the cathartic effects observed. Partial quantitative analysis of the ash gave the following *percentage* results: CaO, 23.76; MgO, 15.62; CO<sub>2</sub>, 13.92; Cl, 11.96; P<sub>2</sub>O<sub>5</sub>, 8.1. Sodium and potassium were present in abundant proportions. The proportion of ash in the fresh tuber was only about 1.4 per cent.

Although it did not seem probable that the observed cathartic action could be due solely to the small amounts of compounds of the alkali earth metals that were present, we tested the effects of the ash in some experiments of which the following, as described in our records, is an illustration.

*Tests of the effects of tuber ash given by mouth to dogs.* Tenth experiment. Weight of the dog, 7.22 kilos.<sup>1</sup> On the first day of the experiment the animal was given 0.6 gram of ash corresponding to the inorganic matter in about 45 grams of tuber. This was administered in several small balls of meat. No other food was given that day. There were no apparent effects. On the following day the fast was continued and an equal portion of ash, i. e., 0.6 gram (which had been largely converted into chlorid by treatment with hydrochloric acid, with subsequent removal of the acid by evaporation to dryness) was dissolved in distilled water and administered by means of a stomach tube. This also failed to produce any noticeable effects. The dog was apparently normal in all respects during the three succeeding days, after which he was no longer kept under observation.

The results of the experiments in this series also fail to explain the effects caused by the tuber. Unfortunately, our supply of tubers was exhausted at this point and further study had to be given up.

#### VII. GENERAL CONCLUSIONS.

Besides yielding the data for composition of *Ibervillea Sonorae*, our study indicates that the tuber does not contain alkaloidal material, or at least not in sufficient proportion to cause particular toxicity. We have not learned the reason for the cathartic effects exhibited by the tuber or its decoctions.

We wish to thank Dr. Gies for his kind guidance and advice in this study, and also for his assistance in carrying out the work.

<sup>1</sup> Daily diet—hashed meat, cracker meal, lard and water, with macerated filter paper to make the feces bulky and approximately normal in consistence. For this purpose bone ash was avoided in order to give the tuber ash full opportunity to display its greatest possible toxicity, without unnecessary interference. A preliminary period of five days established normal conditions.



Fig. A. *Ibervillea Sonoræ* in its native habitat.





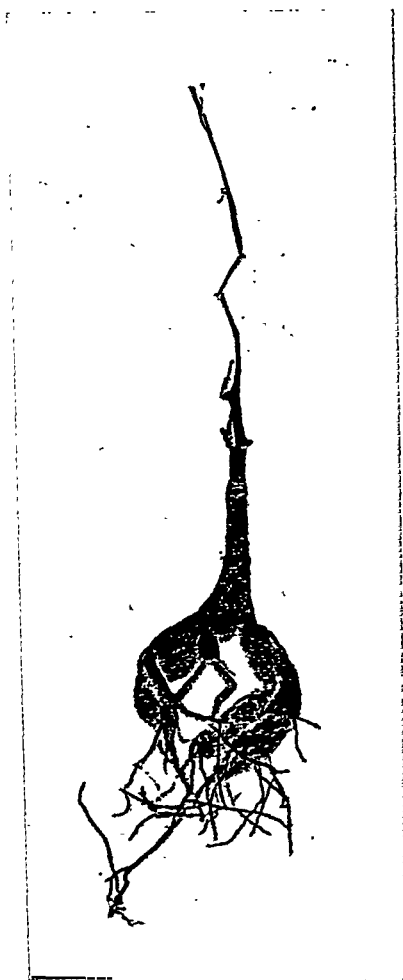


Fig. B. *Ibervillea Sonoræ*, four or five years old.



# THE INHIBITING EFFECT OF POTASSIUM CHLORIDE IN SODIUM CHLORIDE GLYCOSURIA.

By THEO. C. BURNETT.

(From the Rudolph Spreckels Physiological Laboratory of the University of California.)

(Received for publication, October 15, 1908.)

While making experiments on the production of glycosuria by sea-water<sup>1</sup> I became aware that a mixture of sodium chloride and potassium chloride in the proportion of 100 mols. of the former to 2.2 mols. of the latter, was much less effective than pure sodium chloride in inducing glycosuria, and the fact seemed of sufficient interest to warrant further investigation. It has been known for years that the injection of large quantities of sodium chloride will cause glycosuria in rabbits, and Fischer<sup>2</sup> has shown that this glycosuria can be inhibited by calcium chloride. Brown<sup>3</sup> arrived at the same conclusion independently. It seemed important therefore, to ascertain if potassium chloride had also an inhibitory action on the glycosuria caused by the injection of a pure sodium chloride solution.

The technique was the same as formerly, but the well known poisonous effects of potassium precluded the possibility of carrying on the experiments for longer than about three hours, 1 liter (about) of the solution (NaCl, 100 mols. + KCl, 2.2 mols.) being injected during that time.

<sup>1</sup> Burnett, Theo. C.: This *Journal*, iv, p. 57, 1908.

*Note:* Since the publication of the paper just referred to, my attention has been called to an article by Underhill and Closson, *Amer. Journ. of Physiol.*, xv, p. 321, 1906; also to one by Hedon and Fleig: *Arch. internat. de physiol.*, p. 95, July, 1905. Reference should have been made to these in the original article, and I take this first opportunity to correct the omission.

<sup>2</sup> Fischer, M. H.: *Univ. of Calif. Publ. (Physiol.)*, i, p. 87, 1904, where references to earlier authors will be found.

<sup>3</sup> Brown, O. H.: *Amer. Journ. of Physiol.*, x, p. 378, 1904.

When such a solution as the above (sodium chloride plus potassium chloride) is injected into the marginal vein of the ear of a rabbit in large quantities, after about one and a half hours sugar makes its appearance in the urine. The amount varies from a trace to about 0.03 per cent. At first sight this seems like a very marked variation, but by comparing with a pure sodium chloride solution it will be seen that the maximum does not nearly equal the maximum given by Fischer for a pure sodium chloride solution, his tables giving 0.25 per cent.<sup>1</sup> It must be borne in mind that rabbits vary very widely in their susceptibility, and hence the impossibility of having adequate controls in these experiments. I have the records of two experiments in which absolutely no sugar appeared during three hours injection with a mixture of sodium chloride and potassium chloride. On the other hand, one rabbit gave a maximum of 0.04 per cent. The bulk of the experiments, however, gave a figure around 0.01 per cent. When it is said, therefore, that a given solution produces no glycosuria, the fact must not be lost sight of that now and then a rabbit will be found that develops glycosuria, even with Ringer's solution; but in that case it would probably be found that with a mixture of sodium chloride and potassium chloride the amount would be considerably larger, and with a pure sodium chloride solution, a profound glycosuria would result. Table I is selected as fairly typical.

TABLE I.

*December 6, 1907. Belgian hare, female, weight 3000 grams.  
Injection fluid,  $\frac{M}{3}$  NaCl + KCl.*

Time.	Amount injected.	Amount of urine.	
<i>a.m.</i>	<i>cc.</i>	<i>cc.</i>	
8.45			Began injection.
10.00	450	199	Trace sugar.
10.30	150	154	Sugar, .01 per cent.
11.00	200	154	Sugar, .007 "
11.30	150	160	Sugar, .01 "

<sup>1</sup> Fischer, M. H.: *loc. cit.*, p. 111.

The experiment was then tried of inducing glycosuria by a pure sodium chloride solution, and then substituting a mixture of sodium chloride and potassium chloride. The result was a diminution in the amount of sugar, as will be seen from Table II, which is condensed from the original record. It will be noticed that the sodium chloride solution was  $\frac{M}{5}$ , and that sugar appeared in one hour. It seems evident, therefore, that potassium antagonizes, in part at least, the poisonous effects of a pure sodium chloride solution.

TABLE II.

*December 5, 1907. Belgium hare, male, weight 3000 grams.  
Injection fluid,  $\frac{M}{5}$  NaCl: changed later to  $\frac{M}{5}$  NaCl + KCl.*

Time.	Amount injected.	Amount of urine.	
<i>a.m.</i>	<i>cc.</i>	<i>cc.</i>	
9.00			Began injection $\frac{M}{5}$ NaCl.
10.00	375	107	Sugar.
10.30	200	156	Sugar, .027 per cent.
	Changed to $\frac{M}{5}$ NaCl + KCl		
11.00	200	160	Sugar, .016 "
11.30	175	110	Sugar, .014 "
12.00	300	155	Sugar, .007 "

What, now, is the action of calcium chloride under similar conditions? What has been said of potassium applies as well to calcium. When a solution of sodium chloride, 100 mols., plus calcium chloride, 2 mols., is injected in large quantities, a small amount of sugar usually appears in the urine. The maximum obtained was 0.026 per cent—a figure slightly better than for potassium chloride. The minimum, as in potassium chloride, is zero, no sugar appearing in three hours. If, however, the injection is begun with sodium chloride plus calcium chloride and after sugar appears the injection fluid is changed to sodium chloride plus potassium chloride the amount of sugar is increased. The same holds good if the order of the injections is reversed. Table III (p. 354) illustrates this very well.

It will be recalled that Loeb<sup>1</sup> has shown that a pure NaCl solution causes two forms of destructive process in the eggs of the sea

<sup>1</sup> Loeb, J.: *Biochem. Zeitschr.*, ii, p. 81, 1906.

urchin. One of these is the ordinary cytolysis, and the other, a process which he calls "black disintegration." He found that the addition of calcium chloride inhibited the cytolysis, while the addition of potassium chloride inhibited the "black disintegration." In view of this the above results are clear. The addition of one salt, for example, potassium chloride to the injection fluid, prevents one of the destructive processes, with a consequent diminution of the amount of sugar, but not a complete disappearance of it, for there is the other destructive process still active. When sodium chloride plus potassium chloride is substituted for sodium chloride plus calcium chloride, or vice versa, there is an additive effect; either the cells which are partially injured by the

TABLE III.

*August 19, 1908. Belgian hare, female, weight, 1500 grams.  
Injection fluid,  $\frac{M}{8}$  NaCl + CaCl<sub>2</sub>; changed later to NaCl + KCl.*

Time.	Amount injected.	Amount of urine.	
a.m.	cc.	cc.	
9.05			Began injection, NaCl + CaCl <sub>2</sub>
10.05	350	103	Sugar.
11.20	375	363	Sugar, .004 per cent.
	Changed to NaCl + KCl		
12.20	300	279	Sugar, .009 "

one combination, are further injured by the second, or a new lot of cells may be injured by the substituted combination of salts. In either case the result would be an increase in the amount of sugar in the urine. McGuigan and Brooks<sup>1</sup> conclude that "the pathology of experimental glycosuria is very probably due to changes in the protoplasmic activity of the cell." The above results seem to indicate the nature of the change which occurs in the cells involved, be they in the central nervous system, or in the kidney, or elsewhere. Microscopic examination of the kidney in several of these cases shows interesting changes; but the work has not been carried very far, and it will be left for a future communication should results warrant it.

<sup>1</sup> McGuigan and Brooks: *Amer. Journ. of Physiol.*, xviii, p. 256, 1907; cf. also Underhill and Kleiner: *This Journal*, iv, p. 395, 1908.

## THE URIC ACID EXCRETION OF NORMAL MEN.

BY PAUL J. HANZLIK AND P. B. HAWK.

*(From the Laboratory of Physiological Chemistry of the Department of  
Animal Husbandry of the University of Illinois.)*

(Received for publication, October 26, 1908.)

The purpose of this investigation was to observe the course of the excretion of uric acid in normal men living on an ordinary mixed diet. Each subject was allowed to select his own diet, and then was required to ingest the diet selected during the course of six periods of four days each. The variation in the excretion of each individual as well as the average excretion of all the subjects was noted.

Ten university students served as subjects. Quarters were provided where the men could easily be observed as to certain regulations of sleep and diet. The body weights of the subjects ranged from 53.1 to 76.7 kilograms and their ages varied from 19 to 29 years. There were no athletes among the subjects so that no individual took excessive or violent exercise, but all lived the life of the average normal university student.

As was previously stated the diet was a mixed one for all periods. Table I, p. 360, shows the diet for period 1, November 29 to December 2. The diet for the remaining periods was approximately the same in all particulars.

The fruits consisted of red and white cherries, pineapples, apples, oranges, plums, peaches and pears. Of the meats, which were all fresh, there were boiled beef, roast beef, veal, mutton, beefsteak, chicken, pork chops, pork sausage, and boiled and roast ham. The soups consisted of pea, consommé, bouillon and vegetable soup. Cornmeal, rice, cream of wheat, oatmeal, and cracked wheat, composed the cereals. The puddings were custard, starch cream and rice. Ice cream was also served every four days. The vegetables included potatoes, peas, beans, corn and tomatoes. The other constituents were wheat bread, coffee, milk, cocoa, butter and water. Meat was served twice a day, morning and evening. Soups were served at the noon meal only and puddings instead of fruits at night, otherwise the same constituents were served at each meal. In

some instances the men selected no coffee, while in other cases a subject would, for example, drink coffee for the morning meal and cocoa for the evening meal.

For the sake of uniformity in the mode of normal living, each subject was required to carry out a routine daily schedule. They all rose at 6:30 a.m., were immediately weighed and then breakfasted at 7:15 a.m. Lunch was served at 12:15 p.m. At 5:00 p.m. each individual was submitted to a clinical examination. Dinner was served at 6:30 p.m., and all subjects retired between 10:30 and 11:00 p.m. Blood tests, including blood pressure, were made upon each subject at intervals of two weeks. If at any time a subject was found to be abnormal for any reason, the experiment upon him was discontinued until he again became normal. This will be seen to be the case with subjects I and K, Table II, p. 361, where one period for each of these subjects is missing.

The experiment was divided into periods of four days each. Urine was collected in 24-hour periods and preserved with powdered thymol, each bottle being kept in a refrigerator until the entire 24-hour sample had been collected. The urine bottles were then removed, one-half of each sample carefully measured and placed in cold storage. At the end of the four day period, the four one-half volumes were combined to form a single sample for each subject, thus making ten composite samples for analysis. These composite samples were then analyzed for uric acid.

The Folin-Shaffer procedure was employed for the determination of the uric acid.

150 cc. of the urine was introduced into a beaker, 37.5 cc. of the Folin-Shaffer reagent (consisting of 500 grams of ammonium sulphate, 5 grams of uranium acetate and 60 cc. of 10 per cent acetic acid in 650 cc. of distilled water) added and the mixture filtered. 150 cc. of the filtrate was immediately taken, 7.5 cc. of concentrated ammonium hydroxide added and the mixture allowed to stand for 48 hours. The precipitated ammonium urate was then transferred quantitatively to a hardened filter paper and washed with a 10 per cent solution of ammonium sulphate to free it from chlorides. The paper was then removed from the funnel, opened, and by means of hot water the precipitate was rinsed back into the beaker in which the urate was originally precipitated. The volume of the fluid was, at this point, about 150 cc. The solution was allowed to cool to room temperature, 15 cc. of concentrated sulphuric acid was added and the acid solution immediately titrated with  $\frac{N}{20}$  potassium permanganate.

By an examination of Table II, p. 361, it will be observed that there exists considerable uniformity in the daily excretion of



uric acid. Subjects E, F, G, I and J especially exhibited a very constant output. The average daily excretion of subject F, 0.475 gram, was the lowest of any of the subjects. J excreted an average of 0.520 gram per day; I, 0.619 gram and G, 0.644 gram. It might be supposed that differences in the quantity and kind of food taken would influence the daily excretion of uric acid to a marked degree, but it will be seen that this was not altogether the case. Subject F excreted 0.475 gram of uric acid per 24 hours, while subject G excreted 0.644 gram, a much greater quantity than that of subject F, notwithstanding the fact that Table I shows that the diets of subjects F and G were very similar in many respects, the main difference being that G ingested somewhat more meat, potatoes and milk. This shows that in this case the variation in the excretion of the uric acid was probably due entirely to the influence of the diet. The average daily excretion of subject E was 0.660 gram, which was the maximum output of uric acid observed during the experiment, yet the diet of this subject varied but little from that of subjects G and I, the main difference being that E ingested considerably more bread. Subject J differed more from subject E in the matter of diet than either subjects F or G, yet subject J excreted a greater average quantity of uric acid than subject F, but less than subjects E and G. It will be noticed then that individuality in these cases played an important part in the variation of the excretion of uric acid. This variation was probably due to a variation in the endogenous uric acid excretion, which, according to general belief, is governed largely by the individuality of the subject.

Subjects A, B, C, H and K showed less uniformity in the daily excretion of uric acid than the other subjects. Subject A excreted on the average 0.615 gram per day. This subject also showed a gradual increase in uric acid output up to the last period at which time there was a slight decrease. Subject B excreted on the average 0.588 gram with considerable irregular variation. The average excretion of subject C was 0.597 gram with a gradual decrease for the first four periods followed by two periods of higher excretion. Subject H exhibited a peculiarity in that the daily excretion averaged 0.608 gram but varied alternately. In the first period there was a

high excretion, i.e., 0.621 gram, while period 2 was lower, i.e., 0.597 gram. Then again period 3 returned to an excretion of 0.633 gram almost the same as for period 1, and period 4 showed an excretion of 0.583 gram, similar to that of period 2. Beginning with period 1, every alternate period exhibited a striking uniformity in the excretion of uric acid, for example, 0.621, 0.633 and 0.627 gram and 0.597, 0.583, and 0.586 gram for the alternate periods. Subject A showed an average excretion of 0.651 gram with a general tendency for the daily elimination of uric acid to increase. This subject excreted the second largest average quantity of uric acid in 24 hours, although the diet did not differ from that of some of the other subjects except in the large quantity of milk ingested and from the fact that no cocoa nor cereals were taken. The diets of subjects A, B, C and H also were very uniform in quantity as compared with the diets of the other subjects, yet there were variations in the excretions of uric acid, some of the quantities being greater than those of subjects F, G, I and J. Here again, as in subjects E, F, G, I and J, the same reason for this individual variation in excretion may be given. There is probably no other factor which influences these variations to such an extent as does the individuality of the subject.

Concerning the average daily quantity of uric acid excreted by all subjects, it can be readily seen by referring to Table III, p. 364, that it does not amount to 0.7 gram as is generally stated to be the average 24 hour output of uric acid for a normal man. The lowest quantity excreted, 0.475 gram, was by subject F, while the greatest quantity, 0.660 gram, was by subject E. The average for all subjects was 0.597 gram.

The evident tendency of the majority of the subjects to exhibit, for no apparent cause, a relative high uric acid output during the fifth period of the investigation is interesting.

Apart from the uric acid viewpoint our investigation furnished some interesting data relative to the protein ingestion of normal men. Facts regarding this feature may be found summarized in Table IV, p. 365. It will be noted by referring to this table that these ten normal men, ranging in age from 19 to 29 years, when permitted to select their own mixed ration, ingested a diet which contained from 70.3 grams to 109.6 grams

of protein per 24 hours. The average daily ingestion of protein as 91.2 grams. The protein ingested per kilogram of body weight varied from 1.13 gram to 1.51 gram with an average of 1.33 gram.

#### CONCLUSIONS.

1. The average daily excretion of uric acid for ten men, ranging in age from 19 to 29 years, and fed a normal mixed diet was 0.597 gram, a value somewhat lower than the generally accepted average of 0.7 gram for such a period.

2. The average daily protein ingestion for these same subjects, when permitted to select their diet, was 91.2 grams or 1.33 gram per kilogram of body weight.

## Uric Acid Excretion

TABLE I.  
*Diet for Period 1, November 29 to December 2, 1907. Subjects A to K.*

SUBJECT.....	A.	B.	C.	E.	F.	G.	H.	I.	J.	K.
Fruits.....	999.0	995.0	995.0	995.0	995.0	995.0	995.0	995.0	995.0	995.0
Cereals.....	1300.0	1300.0	1300.0	1300.0	1300.0	1300.0	650.0	1300.8	975.6	
Meat.....	820.8	820.8	547.2	820.8	615.6	820.8	820.8	820.8	820.8	
Potatoes.....	840.0	840.0	840.0	840.0	630.0	840.0	840.0	840.0	840.0	568.8
Vegetables.....	225.0	300.0	300.0	300.0	300.0	300.0	300.0	300.0	300.0	368.8
Puddings.....	375.0	375.0	783.0	375.0	375.0	375.0	375.0	375.0	312.6	150.0
Soups.....	608.0	608.0	608.0	608.0	608.0	608.0	356.0	608.0	608.0	343.8
Bread.....	800.0	560.0	720.0	1330.9	480.0	541.7	800.0	698.5	880.0	608.0
Milk.....	2683.2	2683.0	2683.2	2683.2	1238.0	2559.4	4024.8	2683.2	1692.5	876.1
Coffee.....								473.0	472.0	4024.8
Cocoa.....	662.8	662.7	662.7	662.8	662.8	662.8		662.8	662.8	
Butter.....	199.6	252.9	173.7	357.7	293.4	368.7	217.7	77.9	181.5	281.2

TABLE II.  
*Subject A.*

Period.	Date.	Volume of urine.	Specific gravity.	URIC ACID IN GRAMS.		Uric acid nitrogen in 24 hours.
				96 hours.	24 hours.	
		cc.				gram.
I.....	Nov. 29 to Dec. 2	5,660	1,018	2.260	0.565	0.189
II.....	Dec. 3- 6	4,163	1,022	2.216	0.554	0.185
III.....	Dec. 7-10	4,473	1,021	2.324	0.581	0.194
IV.....	Dec. 11-14	4,050	1,0275	2.524	0.631	0.211
V.....	Dec. 15-18	3,705	1,0275	2.864	0.716	0.239
VI.....	Dec. 19-22	5,579	1,018	2.576	0.644	0.215
Total.....		27,630	6,1340	14.764	3.691	1.233
Average.....		4,605	1,0223	2.461	0.615	0.206

*Subject B.*

I.....	Nov. 29 to Dec. 2	3,797	1,023	2.268	0.567	0.189
II.....	Dec. 3- 6	3,604	1,024	2.268	0.567	0.189
III.....	Dec. 7-10	3,389	1,027	2.456	0.614	0.205
IV.....	Dec. 11-14	3,926	1,026	2.336	0.584	0.195
V.....	Dec. 19-22	3,012	1,029	2.400	0.600	0.200
VI.....	Dec. 23-26	3,498	1,028	2.371	0.593	0.198
Total.....		21,226	6,157	14.099	3.525	1.176
Average.....		3,536	1,026	2.350	0.588	0.196

*Subject C.*

I.....	Nov. 29 to Dec. 2	4,939	1,019	2.644	0.661	0.221
II.....	Dec. 3- 6	4,468	1,018	2.304	0.576	0.192
III.....	Dec. 7-10	4,574	1,018	2.300	0.575	0.192
IV.....	Dec. 11-14	4,223	1,0235	2.220	0.555	0.185
V.....	Dec. 15-18	4,623	1,021	2.440	0.610	0.204
VI.....	Dec. 19-22	4,845	1,022	2.416	0.604	0.202
Total.....		27,672	6,133	14.324	3.581	1.196
Average.....		4,912	1,022	2.387	0.597	0.199

## Uric Acid Excretion

TABLE II—continued.

*Subject E.*

Period.	Date.	Volume of urine.	Specific gravity.	URIC ACID IN GRAMS.		Uric acid nitrogen in 24 hours.
				96 hours.	24 hours.	
		cc.				gram.
I.....	Nov. 29 to Dec. 2	8,622	1,010	2.700	0.675	0.226
II.....	Dec. 3- 6	6,720	1,017	2.728	0.682	0.228
III.....	Dec. 7-10	5,942	1,015	2.644	0.661	0.221
IV.....	Dec. 11-14	7,130	1,0155	2.868	0.717	0.240
V.....	Dec. 15-18	6,752	1,016	2.476	0.619	0.207
VI.....	Dec. 19-22	6,419	1,0175	2.412	0.603	0.201
Total.....		41,585	6,0910	15.828	3.957	1.323
Average.....		6,931	1,015	2.638	0.660	0.221

*Subject F.*

I.....	Nov. 29 to Dec. 2	4,482	1,017	1.912	0.478	0.160
II.....	Dec. 3- 6	5,043	1,012	1.824	0.456	0.152
III.....	Dec. 11-14	6,374	1,015	1.884	0.471	0.157
IV.....	Dec. 15-18	6,517	1,014	2.068	0.517	0.173
V.....	Dec. 19-22	7,535	1,0125	1.804	0.451	0.151
VI.....	Dec. 23-26	6,790	1,013	1.904	0.476	0.159
Total.....		36,741	6,0835	11.396	2.849	0.952
Average.....		6,124	1,0139	1.899	0.475	0.159

*Subject G.*

I.....	Nov. 29 to Dec. 2	3,249	1,027	2.824	0.706	0.236
II.....	Dec. 3- 6	3,258	1,025	2.440	0.610	0.204
III.....	Dec. 7-10	2,944	1,029	2.584	0.646	0.216
IV.....	Dec. 11-14	3,160	1,031	2.572	0.643	0.215
V.....	Dec. 15-18	2,806	1,030	2.640	0.660	0.220
VI.....	Dec. 19-22	3,364	1,029	2.388	0.597	0.199
Total.....		18,781	6,171	15.448	3.862	1.290
Average.....		3,130	1,028	2.575	0.644	0.215

TABLE II—continued.  
Subject H.

Period.	Date.	Volume of urine.	Specific gravity.	URIC ACID IN GRAMS.		Uric acid nitrogen in 24 hours.
				96 hours.	24 hours.	
		cc.				gram.
I.....	Nov. 29 to Dec. 2	7,504	1,011	2.484	0.621	0.207
II.....	Dec. 3- 6	6,409	1,012	2.388	0.597	0.199
III.....	Dec. 7-10	6,033	1,014	2.532	0.633	0.211
IV.....	Dec. 11-14	6,090	1,0195	2.332	0.583	0.195
V.....	Dec. 15-18	5,455	1,0205	2.508	0.627	0.209
VI.....	Dec. 19-22	5,732	1,020	2.344	0.586	0.196
Total.....		37,223	6,0970	14.588	3.647	1.217
Average.....		6,204	1,0161	2.431	0.608	0.203

## Subject I.

I.....	Nov. 29 to Dec. 2	5,701	1,013	2.476	0.619	0.207
II.....	Dec. 3- 6	5,083	1,012	2.452	0.613	0.205
III.....	Dec. 7-10	6,373	1,012	2.536	0.634	0.212
IV.....	Dec. 11-14	5,203	1,023	2.456	0.614	0.205
V.....	Dec. 15-18	5,206	1,0175	2.464	0.616	0.206
Total.....		27,566	5,0775	12.384	3.096	1.035
Average.....		5,513	1,0155	2.477	0.619	0.207

## Subject J.

I.....	Nov. 29 to Dec. 2	6,819	1,013	2.060	0.515	0.172
II.....	Dec. 3- 6	5,360	1,016	1.912	0.478	0.160
III.....	Dec. 7-10	5,059	1,018	2.144	0.536	0.179
IV.....	Dec. 11-14	5,179	1,022	2.112	0.528	0.176
V.....	Dec. 15-18	4,446	1,023	2.204	0.551	0.184
VI.....	Dec. 19-22	5,658	1,020	2.048	0.512	0.171
Total.....		32,521	6,112	12.480	3.120	1.042
Average.....		5,420	1,002	2.080	0.520	0.174

## Uric Acid Excretion

TABLE II.—continued.  
*Subject K.*

Period.	Date.	Volume of urine.	Specific gravity.	URIC ACID IN GRAMS.		Uric acid nitrogen in 24 hours.
				96 hours.	24 hours.	
		cc.				gram.
I.....	Nov. 29 to Dec. 2	4,709	1,017	2.152	0.538	0.180
II.....	Dec. 3- 6	3,412	1,025	2.500	0.625	0.209
III.....	Dec. 7-10	4,432	1,020	2.728	0.682	0.228
IV.....	Dec. 11-14	3,632	1,0265	2.536	0.634	0.212
V.....	Dec. 19-22	4,042	1,023	3.104	0.776	0.259
Total.....		20,227	5,1115	13.020	3.255	1.088
Average.....		4,045	1,0223	2.604	0.651	0.218

TABLE III.  
*Average daily uric acid excretion.*

SUBJECT.	Average uric acid excretion (grams per 24 hours).
F.....	0.475
J.....	0.520
B.....	0.588
C.....	0.597
H.....	0.608
A.....	0.615
I.....	0.619
G.....	0.644
K.....	0.651
E.....	0.660
Grand average.....	0.597



TABLE IV.  
*Protein ingestion. Subjects A to K.*

SUBJECT.	Body weight in kilograms.	Daily protein in- gestion in grams.	Daily ingestion of protein per kilo. body weight in grams.
A.....	74.1	97.0	1.31
B.....	65.6	91.4	1.39
C.....	72.2	81.9	1.13
E.....	76.7	109.6	1.43
F.....	53.1	70.3	1.32
G.....	68.7	91.3	1.33
H.....	66.1	99.2	1.50
I.....	63.3	95.6	1.51
J.....	76.0	90.1	1.18
K.....	72.3	85.2	1.18
Average .....	68.8	91.2	1.33



## STUDIES ON ENZYMES: I.

### THE ADSORPTION OF DIASTASE AND CATALASE BY COLLOIDAL PROTEIN AND BY NORMAL LEAD PHOSPHATE.

BY AMOS W. PETERS

*(From the Zoölogical Laboratory of the University of Illinois.)*

(Received for publication, September 18, 1908.)

#### INTRODUCTORY.

Most of the experimental work with enzymes and other similar obscurely known substances is conducted with preparations which contain the desired body in admixture with other, in most cases, undesirable substances. The nature and condition of the extraneous matter is so little known that it is not possible to reproduce with assurance and accuracy the same conditions in the repetition of experiments. The validity and range of application of the results obtained thus remains unknown. Progress of a fundamental nature depends largely upon making some advance in the separation of these substances. The present paper describes the initial steps in an attempt to apply the method of adsorption for this purpose. The phenomena described are usually classed under the term adsorption which is here used, however, only conventionally. In this paper no attempt will be made to explain the real nature of this process which is so typically shown by these experiments.

I take pleasure in acknowledging my obligation to Mr. Bruce M. Harrison, M.S., of the Graduate School of this University, for valuable assistance in obtaining many of the numerical data in the part on the Results of Experiments.

#### SOURCES OF DIASTASE AND CATALASE.

The diastases upon which these experiments were made were purposely produced from materials of different origin. This was done in order that the methods developed might be of general

application, as well as to provide means for a possible future comparison of preparations of diastase of different origins. Germinated wheat, liquid bacterial cultures, and extracts of autolytic liver constituted the principal materials which served as sources of both diastase and catalase.

It is well known that the wheat grain upon germination produces as much or more diastase than barley.<sup>1</sup> The grain was washed with tap water until the wash water was clean. It was then washed with saturated thymol water. It was soaked in distilled water for a day and then placed upon filter paper and kept moist in a closed copper germinating pan. The germination was conducted at room temperature and continued for about three days, after which the cover was removed and the germinated grain permitted to become air-dry for keeping or it was used while still moist. Just before extraction with a liquid as subsequently described the grain was ground in a mill or a mortar.

The liver of cow or pig was purchased fresh in the market, superficially washed and then cut or hacked into small pieces. To one weight of liver there were added three weights of distilled water and a layer of several centimeters in depth of toluol containing 0.5 per cent of thymol was poured on and the whole was then thoroughly agitated. The agitation was repeated on several successive days. For use as a source of diastase and catalase the required quantity of liquid was withdrawn from beneath the toluol layer with a pipette. The content of diastase in the extract of liver so prepared first increased and subsequently disappeared. This material always supplied catalase in great abundance. The liver thus treated was easily kept for months practically free from bacterial action.

The bacterial cultures were grown in a liquid medium of the following composition. Distilled water, 1 liter; monopotassium phosphate, 1 gram; Witte's peptone, 2 to 5 grams; Kahlbaum's soluble starch, 2 to 5 grams. The whole was sterilized by steam at 100° C. in a Jena glass Erlenmeyer flask in which the culture was subsequently raised. The natural reaction of the liquid was permitted to remain. In this medium numerous kinds of bacteria grew abundantly both at room temperature and especially

<sup>1</sup> Maerker-Delbrück: *Spiritusfabrikation*, pp. 106, 287, 1903.

upon incubation at  $37^{\circ}\text{C.}$  to  $40^{\circ}\text{C.}$  The first material for inoculation was obtained from the water and from the decaying vegetable matter of a small, much polluted stream. The bacteria so obtained were found to be diastatic but the great variety of species was undesirable. By plating upon agar, pure cultures of a diastatic organism were obtained which were used in the experiments to be described. The catalase content of these cultures would develop gradually and reach its maximum after the amount of diastase had become too small to be of good service experimentally.

1

#### IDENTIFICATION OF DIASTASE.

The presence and concentration of diastase were always tested by its effect upon a freshly-prepared 1 per cent solution of Kahlbaum's soluble starch. The starch was dissolved in hot water and a few crystals of thymol added before cooling. Such a solution as a rule contained no perceptible amount of reducing sugar. In testing for diastase one volume of starch solution was thoroughly mixed with an equal volume of the fluid to be tested, toluol containing 0.5 per cent thymol added to a depth of several centimeters, and the mixture incubated at  $50^{\circ}\text{C.}$  for periods varying from one to twenty-four hours. Sugar was estimated both at the beginning and at the end of the fermentation period and frequent controls showed the absence under these conditions of hydrolysis due to other influences than the diastase.

Sugar was estimated by Bang's method,<sup>1</sup> chosen on account of its convenience and accuracy. The method is based upon the reduction of a copper solution in the presence of potassium sulphocyanide, the excess of unreduced copper being estimated by titration with standard hydroxylamine solution. In my determinations, only 2 cc. of the sugar solution were used and hence only 10 cc. of the copper solution. All of the precautions suggested by Bang were observed. The results are expressed as volumes of hydroxylamine solution used in the titrations, and as their equivalents in milligrams of dextrose per cubic centi-

<sup>1</sup> Bang: Zur Methodik der Zuckerbestimmung, *Biochem. Zeitschr.*, ii, pp. 271-290, 1907.

meter of the fermenting solution. The nature of the reducing substances produced by the enzymes studied was not investigated.

As a unit for the measurement of diastase, the production of 0.1 milligram of sugar during the first hour of digestion at 50° C. was arbitrarily chosen. This amount is equivalent to nearly 0.3 cc. of the standard hydroxylamine solution and lies outside of the limits of experimental error.

In testing a bacterial culture for its content of diastase the mode of procedure was as follows: To a measured volume of the culture fluid (10 to 100 cc.) contained in a tall cylinder, about one-fifth volume of toluol was added and the mixture thoroughly shaken. Further bacterial action was thus inhibited. A volume of 1 per cent soluble starch equal to the volume of culture fluid used was then added. After shaking and withdrawing a portion in which the original sugar content of the mixture was estimated, the mixture was incubated at 50° C. Portions of the mixture were withdrawn at intervals in order to test the progress of digestion. The amount of culture fluid used should be such that an appreciable amount of sugar (by Bang's method) is formed during the first hour. A control test was made in which boiled culture was used. It may here be stated that in the absence of toluol, digestion with the bacterial culture resulted in the disappearance of the starch as measured by the iodine reaction but no sugar could be detected.

#### IDENTIFICATION OF CATALASE.

The term catalase has been used to denote a substance of protoplasmic origin which produces evolution of oxygen from hydrogen peroxide, whose activity is inhibited at or below a boiling temperature, and which when free from peroxidase does not give the guaiacum and similar color reactions. The unity of this substance or the reality of its enzymic nature were not subjects of investigation. The presence of catalase was determined by treating the liquid under examination with an equal volume of approximately 3 per cent neutralized hydrogen peroxide. To facilitate detection of small volumes of gas formed under these conditions it was often found desirable to pour strong alcohol on the surface of the mixture. Bubbles of gas which may

otherwise be obscured by the turbidity of the fluid become easily visible in passing through the clear, alcoholic layer.

#### SEPARATION OF ENZYMES.

Isolation and purification of enzymes employed in my experiments were accomplished by the use of two procedures, viz: extraction by acetone and by adsorption. By means of extraction with 50 per cent acetone, i.e., the addition of one volume of pure acetone, it is possible to free the fluids which have served as our sources of enzymes from contaminating proteins, carbohydrates and other extraneous matter without affecting the activity of the enzymes diastase and catalase. The resulting solution usually gives no reaction with tannic acid. By this means, however, soluble crystalline material may fail to be removed and hence adsorption of the enzymes by a number of substances has been made use of in their isolation in a state of still greater purity.

Experiments have been made to ascertain the influence of 50 per cent acetone upon the diastase and catalase contained in germinated wheat, autolyzed liver tissue and bacterial cultures.

*Germinated wheat.* A clear aqueous extract of dried germinated wheat contained both diastase and catalase. A similar extract made with 50 per cent acetone was found to contain only diastase. Throughout these tests it was uniformly found that catalase was insoluble in this fluid. Residues from the extraction with acetone possessed catalytic activity, hence the ferment is not destroyed by this reagent. Furthermore, on mixing some of the catalytically active residue with a mixture of equal volumes of hydrogen peroxide and acetone, oxygen is evolved in large amounts. Failure to detect catalase in 50 per cent acetone extracts must therefore have been due to its insolubility in this medium.

*Autolyzed liver tissue.* When liver tissue cut in small pieces is allowed to autolyze in the presence of water and toluol containing 0.5 per cent of thymol, a solution results which possesses marked catalytic power. During the earlier stages of self digestion it also contains diastase. If such an extract is mixed with an equal volume of acetone a heavy precipitate results, the filtrate from which contains no catalase. It does however contain appreciable

amounts of diastase, sometimes a considerable quantity. As a rule the diastatic power of such a filtrate is not equivalent to that of the original solution. The experiments on germinated wheat showed that diastase was soluble in 50 per cent acetone and it seems evident, in view of the adsorption experiments to be described later, that diastase is carried down with the precipitate caused by the acetone. Diastase will therefore be contained in the filtrate only when this precipitate is not sufficient in bulk to adsorb all of it. These facts have suggested the possibility of separating enzymes from their solutions by the addition of a protein such as Witte's peptone after treatment with acetone. Such a method will be described later.

*Bacterial cultures.* In the separation of the enzymes from bacterial cultures, filtration is ineffective because of the fact that the enzymes are apt to be retained by the filter. Centrifugation is difficult and at best affords incomplete separation. It has been found however that when an equal volume of acetone is added to a bacterial culture, and allowed to act on the bacterial cells for some time before it is removed in great part by evaporation under an air blast at room temperature or at 40° C. the resulting unfiltered fluid possessed greater diastatic activity than the original culture. This may have been due to the action of the acetone upon the bacterial cells in killing them and rendering them more permeable to their own enzymes. From a fluid so obtained more of the ferment could be obtained by the adsorption methods to be described than from the original fluid. This method may have extended application in the extraction of bacterial cells for other purposes.

#### ADSORPTION OF THE FERMENTS.

*Witte's peptone.* By the addition of Witte's peptone in 10 per cent watery solution to a solution containing diastase or catalase and subsequently adding an equal volume of acetone a precipitate will be obtained in which the ferments are concentrated. I have made use of this observation in the methods employed in these experiments. As a reagent 20 grams of Witte's peptone were dissolved in 200 cc. of hot water. It should not be boiled but the heat should be great enough to destroy any ferments



which might be present. After cooling an equal volume of acetone is added, the abundant precipitate allowed to subside and the supernatant fluid removed by decantation. To the precipitate is added enough 50 per cent acetone to make the mixture up to the original volume, i.e., 200 cc. This reagent, a 10 per cent suspension of precipitated peptone in 50 per cent acetone, is applied for the adsorption of diastase and catalase from liquids containing them. Such liquids are treated with equal volumes of acetone and 10 cc. of the peptone suspension, containing 1 gram of peptone, are added. The precipitate with the adsorbed enzyme, is removed by decantation or by the centrifuge. It may be washed with 50 per cent acetone though this is usually unnecessary. If the separation of the enzyme is not quantitative the process can be repeated until all of the enzyme is so removed. The precipitate can then be digested in water and the action of the adsorbed enzymes tested.

This method is of advantage not only because it concentrates the enzymes but especially because it removes such impurities as may be precipitated by 50 per cent acetone and such soluble crystalline substances as are not absorbed by the peptone. The enzyme separated by this method, is however associated with the peptone from which it is indistinguishable.

*Lead phosphate.* The lead phosphate reagent used in this method was prepared by precipitating 100 cc. of 0.3 M solution of lead acetate with 100 cc. of 0.2 M solution of sodium phosphate, washing the precipitate thoroughly with water and finally suspending it in 500 cc. of water. According to Dammer<sup>1</sup> normal lead phosphate does not hydrolyze in cold water and only slowly in water at 50° C.

For adsorbing the enzymes studied in these experiments, one volume of this suspension of lead phosphate was added to the fluid containing diastase, either aqueous or containing 50 per cent of acetone. A more concentrated suspension of lead phosphate may be used if dilution is to be avoided. The advantage which this reagent possesses over the Witte's peptone is that it favors more complete purification of the enzyme. The insolubility, real or supposed, of the enzymes in condition of adsorption does not prevent their action upon their appropriate substrata.

<sup>1</sup> *Handb. d. anorg. Chem.*, iv., p.560, 1903.

The methods described in this paper for obtaining diastase and catalase from different types of materials may be briefly summarized as follows:

Solid materials, e.g., germinated wheat or autolyzed liver, are extracted with water, toluol-thymol being used as an antiseptic. An equal volume of concentrated acetone is added to the extract and the mixture warmed at  $40^{\circ}$  C. for a short time. A heavy precipitate formed under these conditions is apt to contain most of the enzymes, diastase and catalase. If no precipitate forms, the enzymes are absorbed from the fluid by means of Witte's peptone suspended in 50 per cent acetone or by normal lead phosphate suspended in water.

Liquid bacterial cultures are treated with equal volumes of acetone. If lead phosphate is used as the adsorbent, it is added to the mixture in watery suspension after the bulk of acetone has been removed by evaporation. For the peptone method acetone is again added in the same quantity (or it is not removed at all) and the mixture finally treated with a suspension of Witte's peptone in 50 per cent acetone.

#### RESULTS OF EXPERIMENTS.

The experiments described in this section illustrate the application of these methods in a variety of conditions.

##### *Adsorption by Lead Phosphate.*

Ten cc. of an extract of germinated wheat made with 50 per cent acetone were thoroughly mixed with 10 cc. of lead phosphate suspension. The lead phosphate (with its adsorbed enzymes) was removed by the centrifuge and made up to 10 cc. with distilled water. This suspension, designated as Precipitate 1, was mixed with 10 cc. of a 1 per cent soluble starch solution, and incubated at  $50^{\circ}$  C. after estimating sugar in the mixture by Bang's method.

The centrifugate obtained above was treated with the lead phosphate obtained by centrifuging 10 cc. of the watery suspension of lead phosphate used as adsorption reagent, and the mixture made up to 20 cc. with distilled water. After thorough shaking, the lead phosphate (with its adsorbed enzyme) designated as Precipitate 2, was removed by the centrifuge, made up to 10 cc. with water, mixed with an equal volume of a 1 per cent solution of soluble starch and, after estimation of its sugar content, incubated at  $50^{\circ}$  C.

Sugar estimations were made at the end of 18 hours and at the end of 24 hours incubation.

The results are given in the following table:

TABLE 1.

	VOLUME OF HYDROXYLAMINE.				SUGAR.
	Initial reading.	After 18 hours of incubation.	After 24 hours of incubation.	Hydroxyl-amine difference.	
	cc.	cc.	cc.	cc.	mg./cc.
Precipitate No. 1.....	7.1	4.7	4.7	2.4	1.0
Precipitate No. 2.....	7.55	7.1	7.1	0.45	0.2
Filtrate No. 2.....	6.4	6.4	6.4		

These results show that the first treatment with lead phosphate resulted in the adsorption of about five-sixths of the diastase, the remainder being removed by the second addition of lead phosphate.

It is probable that a given quantity of lead phosphate has its characteristic saturation capacity and hence to remove all of the enzyme from a fluid it may be necessary to treat it more than once with the adsorbent. The relation between quantity of adsorbent and quantity of enzyme requires further investigation than has here been given it. Similar results to those given above have frequently been obtained and show the capacity of lead phosphate to adsorb diastase completely and in an active condition.

The next series of experiments showed the power of lead phosphate to adsorb diastase from extremely dilute solutions.

One cc. of the acetone extract of germinated wheat used above was diluted to 100 cc. with water, 10 cc. of lead phosphate suspension were added, the mixture thoroughly shaken, and after settling, the supernatant fluid was decanted. The precipitate was made up to 10 cc. with water, mixed with 10 cc. of 1 per cent soluble starch, sugar content estimated and incubated at 50°. The hydroxylamine values were as follows:

Before incubation, 8.3 cc.; after 3 hours, 6.4 cc.; after 5½ hours, 6.1 cc.; after 23 hours, 6.1 cc. The hydroxylamine difference is thus 2.2 cc. which represents very nearly 1 mg. of sugar per cc. that was produced by the

adsorbed diastase. Negative results were obtained when the filtrate from the lead phosphate was similarly tested.

A series of experiments to determine the possible effect of lead phosphate upon diastatic action was made as follows:

Six cc. of lead phosphate suspension were added to 4 cc. of a 50 per cent acetone extract of malted wheat. After thorough shaking, 10 cc. of 1 per cent soluble starch solution was added after estimation of sugar in a portion of the mixture, the rest was incubated at 50° C. for 48 hours. As a control, a mixture of 4 cc. of the same extract, with 6 cc. of water and 10 cc. of soluble starch was similarly incubated. The results follow:

TABLE 2.

COMPOSITION.	VOLUME OF HYDROXYLAMINE.				SUGAR.
	Initial reading.	After 48 hours.	Hydroxyl-amine difference.	Acceleration.	
	cc.	cc.	cc.	cc.	mg./cc.
4 cc. extract of wheat 10 " per cent starch 6 " $\text{Pb}_3(\text{PO}_4)_2$	4.7	0.9	3.8	1.7	0.7
4 " extract of wheat 10 " 1 per cent starch 6 " distilled water	4.2	2.1	2.1		

The results show that the action of diastase is markedly accelerated by the presence of lead phosphate. The possible causes of this acceleration have not been studied.

The diastase contained in an extract of autolyzed liver tissue may similarly be adsorbed by lead phosphate as the following experiment indicates:

Fifty cc. of aqueous extract of liver tissue which had been allowed to undergo 10 days autolysis was mixed with 50 cc. of lead phosphate suspension. After shaking thoroughly and allowing the precipitate to settle 75 cc. of supernatant fluid were removed by decantation and the residue (25 cc.) made up to 50 cc. with a 10 per cent solution of soluble starch. This was digested at 50°, portions being withdrawn for sugar estimation at the end of 1½, 3 and 21 hours. The hydroxylamine differences were 0.4, 0.7, and 1.4 cc., respectively. The last value is equivalent to about 0.6 mg. of sugar per cc.

The following experiment indicates the applicability of the method in the removal of diastase from bacterial cultures.

One hundred cc. of the bacterial culture were thoroughly shaken with 10 cc. of toluol and 83 cc. of the aqueous fluid removed by a siphon. To this was added an equal volume of lead phosphate suspension. On separation of the precipitate from the supernatant fluid by decantation, both were found to contain diastase. If however the lead phosphate precipitate is collected upon a filter paper by means of a Buchner funnel and suction and the supernatant fluid passed through the layer of lead phosphate on the paper, the latter takes up practically all of the diastase. The results given below show that this is the case. The precipitate with the filter paper was suspended in water and 1 per cent soluble starch added in such proportion that the volume of the mixture was the same as that of the original culture and contained 0.5 per cent of soluble starch. The filtrate was mixed with an equal volume of 1 per cent soluble starch and hence the fluid is twice as dilute as the original culture.

TABLE 3.

*Precipitate.*

Duration of digestion, hours.	Hydroxylamine difference.
0	0
3½	1.5
15.7	2.0

*Filtrate.*

Duration of digestion, hours.	Hydroxylamine difference.
0	0
3½	0
16	0.2

These results show that this procedure was successful in removing practically all of the diastase from the culture fluid.

The power of some other substances to adsorb diastase and catalase in a similar fashion has been tested. Zinc phosphate was found to be less efficient in adsorbing diastase and possessed no power of increasing its activity as did lead phosphate. Precipitated gelatinous aluminum phosphate was less active than zinc phosphate. Zinc oxide used in the same manner gave precipitates and filtrates which were wholly inactive.

The following experiments represent attempts to remove the enzyme from the lead phosphate after adsorption.

The diastase from 10 cc. of a strongly diastatic extract of germinated wheat was adsorbed by means of lead phosphate, the latter was thoroughly washed with water, made up to 20 cc. with water and 20 cc. of 0.002 per cent phosphoric acid added. The mixture was allowed to stand for two days at room temperature: at the end of this time, 10 cc. of the clear, acid supernatant fluid was withdrawn and tested for diastase with negative results. The lead phosphate in this mixture when washed free of acid was found to be strongly diastatic. The failure of acidified water to dissolve the diastase may possibly be connected with the inhibitive influence which the acid of the medium would exert upon the very small hydrolysis of normal lead phosphate at room temperature.

The power of alkalis to remove the enzyme from the adsorbent was not tried on account of the well-known power of alkalis to inactivate diastase.

Distilled water and 50 per cent acetone were tested in this regard. The results showed that appreciable amounts of diastase could be extracted from the lead phosphate precipitate by distilled water; 50 per cent acetone was found to be about one-half as active as water in this respect. Fifty per cent glycerine was also found to be an excellent solvent and preservative. It should be noted in this connection that the purer an aqueous solution of diastase is, the greater is its instability and this fact may have a bearing upon the above experiments. Since lead phosphate adsorbs the enzyme in a very active condition there is for many purposes no need of its solution. The preparation of the thus far imaginary pure diastase was not sought in these experiments.

#### *Adsorption by the Proteïn Method.*

The following experiments illustrate the application of the method of adsorption of diastase by peptone.

An active aqueous extract of germinated wheat was prepared and filtered. 25 cc. of acetone were added to 25 cc. of the extract, no precipitate being formed. 10 cc. of the peptone reagent was added and after standing for an hour with occasional shaking the precipitated peptone was removed by the centrifuge. Both precipitate and supernatant fluid (designated filtrate) were tested as follows: The precipitate, treated with 25 cc. of

water and mixed with 25 cc. of 1 per cent soluble starch, was incubated at 50° for 1 hour. At the end of this time the sugar estimation showed an hydroxylamine difference of 2.9 cc., equivalent to 1.2 mg. of sugar per cc. 20 cc. of the filtrate was mixed with 20 cc. of 1 per cent soluble starch which made the fluid containing the enzyme about 5 times as dilute as the original extract. Sugar estimation in this digestion mixture gave an hydroxylamine difference of 2.8 cc. in the first hour, showing that a large portion of the diastase had not been adsorbed. By repeated application of the peptone reagent practically all of the diastase could have been removed from the original extract.

Another experiment illustrates the removal of diastase by this method from an extract of autolyzed liver tissue.

Fifty cc. of an extract of liver tissue autolyzed for 7 days was mixed with 50 cc. of acetone and warmed at 40° for 15 minutes. The precipitate so obtained was filtered off, suspended in distilled water and warmed at 40° for 3 hours. At the end of this time the insoluble matter, designated below "Residue" was filtered off. The filtrate from the residue is designated the "Digest." The "Residue" was mixed with distilled water and starch solution so that the volume of the mixture was the same as the volume of the original liver extract. A portion of the "Digest" was mixed with starch solution in proportion to make the dilution 3 compared with the original liver extract. Another portion of the "Digest" was mixed with lead phosphate reagent, the final dilution of the diastase in this mixture also being 3. All were incubated at 50° with the following results:

TABLE 4.

*Residue.*

Duration of digestion, hours.	Hydroxylamine difference, cc.
0	0
1	0.7
3½	1.3

*Digest.*

Duration of digestion, hours.	Hydroxylamine difference, cc.
0	0
1	0.2
5	0.4

*Lead Phosphate.*

Duration of digestion, hours.	Hydroxylamine difference, cc.
0	0
1½	0.2
6½	1.0

These figures show that much diastase remained unextracted in the residue and that such quantity as is extracted is easily concentrated by lead phosphate, thus showing that the two processes can be used together. The accelerating effect of the lead phosphate is of course represented in the above figures.

The method as applied to a bacterial culture is illustrated by the following experiment:

Twenty-two cc. of a perfectly clear fluid very rich in diastase was removed by decantation from a 7 days' old bacterial culture. 22 cc. of acetone were added, without forming a precipitate. 6 cc. of peptone reagent were then added, and the precipitated peptone removed by the centrifuge. The fluid was again treated with 6 cc. of peptone reagent, and the precipitated peptone removed by the centrifuge. Each of these precipitates was digested in 22 cc. of water and 22 cc. of 1 per cent soluble starch solution. Sugar estimations after incubation at 50° gave the following results:

TABLE 5.  
*Precipitate 1.*

Duration of digestion, hours.	Hydroxylamine difference.
0	0
0.6	0.7
1	0.9
11.5	3.2

*Precipitate 2.*

Duration of digestion, hours.	Hydroxylamine difference.
0	0
1	0.1
8	1.1

#### QUESTIONS FOR FURTHER INVESTIGATION.

There are important aspects of the method of adsorption and also of uses to which it might be applied that have not been developed in this paper. Among these may be mentioned its quantitative relations, such as the saturation capacity of a given amount of adsorbent, also the quantitative separation of the enzymes. Further questions pertain to the purity of the enzymes adsorbed and the feasibility of studying their properties as they are exhibited by the adsorbed substance. There also remains the fundamental question of the real nature of the process here only conventionally denominated as adsorption.



in two fractions; one at 100° and 10 mm. pressure, the other at 200° and 10 mm. pressure. The distillate obtained in the first fraction was saponified by prolonged boiling in water. The distillate secured in the second fraction was saponified by heating in baryta water, the barium removed with sulphuric acid. These two solutions of amido-acids were then concentrated at 50° and a pressure of 10 mm. until the first signs of crystallization appeared at room temperature; that is, concentrated to saturated solutions. Three hundred and seventy-five grams of arginin, prepared from previous lots of salmin, were then converted into the carbonate according to the method of the Kossel school, and a saturated solution formed therefrom. In such a solution are represented three states of arginin, in equilibrium; the carbonate, bicarbonate and hydroxide. These three solutions were then mixed. From this solution, which formed the substrate of the experiment, two small controls were prepared: one consisting of the plain solution; the second consisting of an equal amount, to which had been added some of the glycerin extract that had been inactivated by heating. The remaining bulk of the solution was divided into two parts. To one, representing half of the material, was added 100 cc. of a fresh glycerin extract of the livers of the variety of clam used in the previous experiment. To the other lot was added 2 grams of pancreatin (Grübler's Pancreatin nach Spateholz). To each, toluol was added in large amount, and the four tests then set aside for nearly four months at room temperature, sealed.

As time passed cloudiness developed in the two larger tests; the two controls remained clear. At the end of nearly four months, the containers were opened and the contents tested bacteriologically, with negative results. All the four solutions were then diluted with four volumes of water, acidulated with sulphuric acid and mixed with three volumes of absolute alcohol. Heavy white precipitates occurred in the two larger tests; none in the controls.

The precipitate in the test with the glycerin extract of the clam liver was washed with alcohol, redissolved in water, precipitated and washed with alcohol, again dissolved in water and a third time precipitated and washed with alcohol. Finally it was dissolved in water, precipitated as the picrate and again recovered

according to the Kossel method for the purification of salmin. It was finally again precipitated from solution in water by alcohol, washed with alcohol, then absolute alcohol, then ether, the ether removed with a stream of dry air, and the powder dried *in vacuo* over sulphuric acid for a week, then dried for several days *in vacuo* at  $105^{\circ}$ . It was a white, rather fluffy powder, and weighed 5.3 grams. This powder was soluble in thirty parts of water at room temperature, was salted out of solution by saturation with ammonium sulphate and by 10 per cent concentration of sodium chloride. On mixing with blood serum, a heavy colloidal precipitate was produced. It was easily digested with trypsin, but was entirely resistant to pepsin. One gram of the powder, on hydrolysis with hydrochloric acid, yielded 0.89 gram of arginin. The elementary analysis of the powder gave the following figures:

0.3402 gm. of substance yielded 0.5028 gm.  $\text{CO}_2$  and 0.2051 gm.  $\text{H}_2\text{O}$ .

0.3024 gm. of substance yielded 0.079 gm. of nitrogen.

0.5400 gm. of substance yielded 0.2659 gm. barium sulphate.

There are two slightly varying formulae ascribed to salmin, based upon different analyses by different men. I tabulate them as follows:

Piccard.....	$\text{C}_{16}\text{H}_{32}\text{N}_9\text{O}_4$ .
Piccard.....	$\text{C}_{30}\text{H}_{57}\text{N}_{17}\text{O}_6$ .
Cloetta.....	$\text{C}_{30}\text{H}_{57}\text{N}_{17}\text{O}_6$ .
Schmiedeberg.....	$\text{C}_{16}\text{H}_{29}\text{N}_9\text{O}_2$ .
Kossel.....	$\text{C}_{16}\text{H}_{31}\text{N}_9\text{O}_3$ .
Kossel.....	$\text{C}_{30}\text{H}_{59}\text{N}_{17}\text{O}_7$ .
Taylor.....	$\text{C}_{16}\text{H}_{31}\text{N}_9\text{O}_3$ .
Taylor.....	$\text{C}_{16}\text{H}_{29}\text{N}_9\text{O}_2$ .

The figures of Goto<sup>1</sup> agree with none of these fully, best with the last figure of Kossel. The variations in the hydrogen and oxygen may be disregarded, the real difference is in the ratio of carbon to nitrogen. This ratio is for the formula  $\text{C}_{16}\text{H}_{29}\text{N}_9\text{O}_2$ , 1777:1000; for the formula  $\text{C}_{30}\text{H}_{59}\text{N}_{17}\text{O}_7$ , 1765:1000. If the figures for the elementary analysis are to be contrasted with the figures demanded by these two equations, the larger formula must be written with the same relations in the hydrogen and oxygen that obtain in the smaller. Then we have:

<sup>1</sup> Goto: *Zeitschr. f. physiol. Chem.*, xxxvii, p. 94.

	Calculated for $C_{16}H_{29}N_9O_2 \cdot H_2SO_4$ :	Found:	Calculated for $C_{30}H_{53}N_{17}O_4 \cdot 2H_2SO_4$ :
C.....	40.25	40.3	39.52
H.....	6.50	6.7	6.25
N.....	26.41	26.2	26.12
$H_2SO_4$ .....	20.55	20.7	21.51

The figures present a satisfactory agreement with those demanded by the first equation. During the course of the experiment therefore there has been formed from the amido-acids a substance having the percentage composition of salmin. This substance has the common reactions of salmin, though these are of little value as criteria for the question under consideration. A specific reaction for salmin is not known. From the standpoint of the physical chemist there can be no question that in view of the theory of reversed reactions, the substance might without further investigation be denominated as salmin. From the point of view of organic chemistry, however, the qualitative demonstration in the strictest sense has not been accomplished; it has not been shown that the substance obtained by synthesis is the same in *molecular construction* as that natively residing in the spermatzoa of the salmon.

This aspect of the question demands particular attention in view of the fact that in the reported syntheses of disaccharides from hexoses under the action of ferments, the iso-sugars have been obtained, not the natural disaccharides from which the primary sugars were derived. There can be no question that in these several investigations disaccharides have been synthesized from monosaccharides, but there has been a qualitative deviation. In the language of Wegschneider, there has been not only a "Folgewirkung" but also a "Nebenwirkung." In view of the little we know of the iso-sugars, these reversion experiments urgently require repetition and amplification. But there can be no question, in view of the results of the elementary analyses that disaccharides have been formed from monosaccharides through the action of inversion-ferments.

Salmin is composed of four amido-acids, as described by Kossel: arginin, serin, prolin, and amidovalerianic acid. If the molecule of salmin contained but one molecule each of the four named amido-acids (disregarding the stereoisomerism of the amido-acids) it is evident that twelve isomeric molecular forms

would be possible. Each molecule of salmin, however, contains not one molecule each of the component amido-acids, but several. Kossel and Dakin<sup>1</sup> have suggested two formulæ:

- 10 mol. arginin + 1 mol. amidovalerianic acid + 2 mol. prolin + 2 mol. serin;  
 12 mol. arginin + 1 mol. amidovalerianic acid + 3 mol. prolin + 2 mol. serin.

In another paper<sup>2</sup> I suggest another equation, very like those of Kossel and Dakin:

- 12 mol. arginin + 1 mol. amidovalerianic acid + 2 mol. prolin + 3 mol. serin.

The number of isomers in such an equation would run into the hundreds. The investigations of the Fischer school have taught us that in the study of the polypeptides, the condensation-products of amido-acids that so much resemble peptones, it is possible to determine to some extent the relations in the linkings in the chains of amido-acids. These methods are not at present highly enough developed to be applied to the study of protamins; we know nothing of the linkings of the amido-acids in their molecules. Since we do not know the internal structure of the molecule of salmin as it occurs in nature, we cannot determine whether the substance of the same percentage composition and generally known reactions obtained in the experiment herein reported is identical with the salmin that occurs in the spermatazoa of the salmin or an isomer. There can be no question that a protamin has been synthesized; the qualitative study of the product must however await new and exact knowledge of the molecular structure of natural salmin. I believe it to be the same protamin, there are no apparent reasons to doubt it; but it must be admitted that from the standpoint of the strict organic chemist it is not now possible to prove that in this molecule exist the same linkings of amido-acids that exist in the natural protamin in the spermatozoa of the salmon. The velocity of the reaction is low, the yield very small—as has been the fact with all ferment reversions. The remainder of the material will be preserved for future investigations.

<sup>1</sup> Kossel and Dakin: *Zeitschr. f. physiol. Chem.*, xli, p. 407, 1904.

<sup>2</sup> Taylor: *This Journal*, v, p. 394, 1909.

The material precipitated by alcohol in the test with bovine pancreatin is not a protamin. The amount was 3.8 grams. It is salted out of watery solution by saturation with ammonium sulphate, but not by a 10 per cent concentration of sodium chloride. It is not only digestible with trypsin, but also by pepsin as well. The analysis for nitrogen indicates that the substance cannot be a protamin.

0.3229 gram of substance yielded 0.0716 gram of nitrogen = 22.2 per cent.

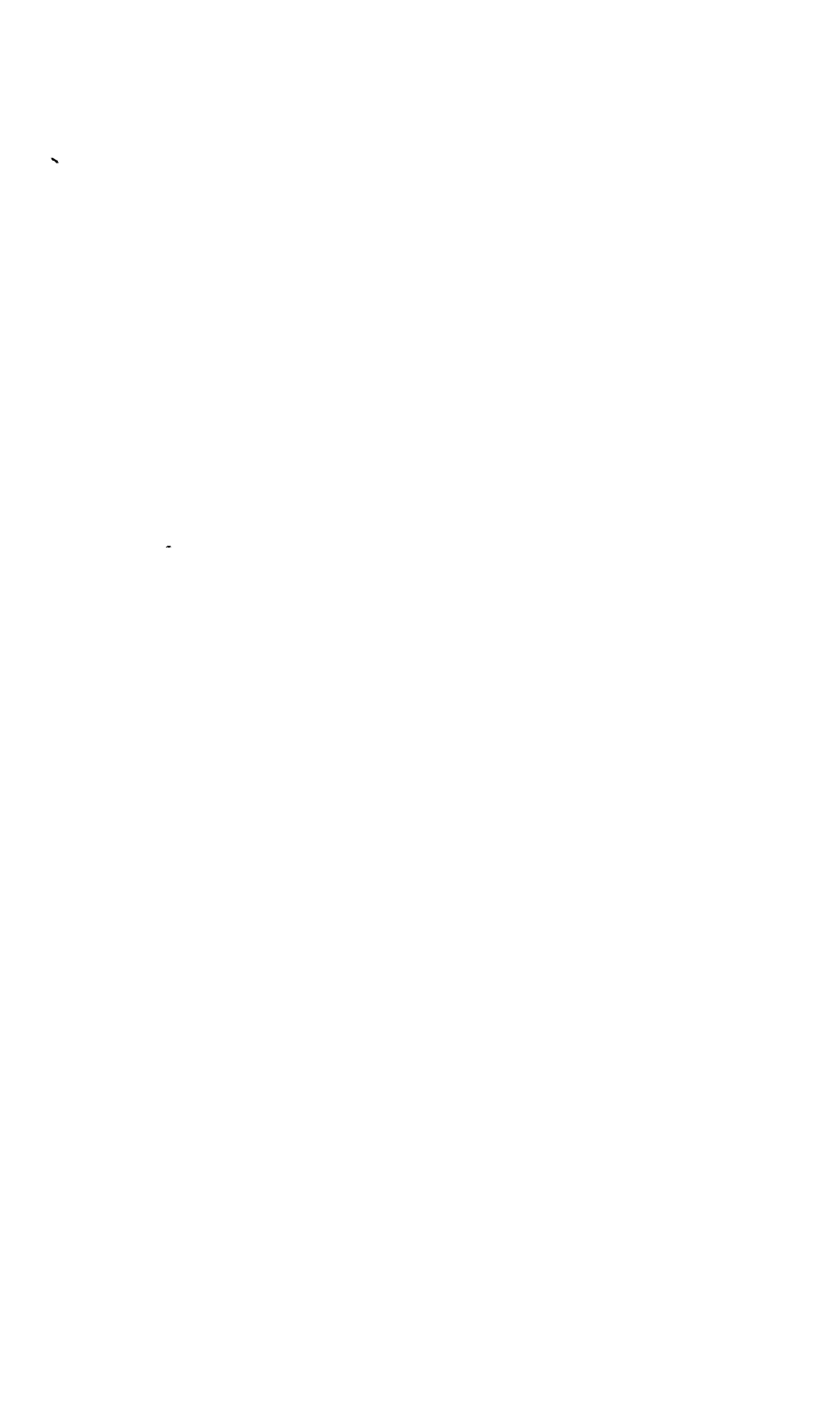
This figure is far too low for salmin sulphate, being much nearer that of arginin sulphate. This substance is not, however, to be derived from the preparation of pancreatin used in the experiment. One and a half grams of this pancreatin dissolved in 60 parts of water and mixed with 3 volumes of alcohol will not give a heavy precipitate. Nevertheless, in the future, fresh pancreatic juice alone is to be employed. I have done nothing further with this material. Rather than consume it in analytical studies that would in all probability reveal nothing, I shall reserve it for the following future experiment: the reintroduction of the material into a saturated solution of the amido-acids concerned, in the presence of fresh trypsin. This experiment ought in my opinion to be done with the iso-maltose and iso-lactose; they should be placed in strong solutions of the appropriate hexoses, with fresh portions of the inversion-ferments to determine whether further changes would occur.

Additional investigations with the ferments in the liver of the clam have yielded interesting results. The enzymic strength of different glycerin extracts varies greatly at different times, with different collections, and at different seasons of the year. The habits of the clams are so little understood that it is not possible to attempt to relate the variations to any variables in their habits of life. Certain is one fact: that at the times when the glycerin extract is quite inert, examination of the digestive apparatus will indicate that the animal is fasting. There are great seasonal variations in the condition of the clams; in the summer they are usually fat and large, in the winter often poor and thin. Investigations of the glycerin extract have also elicited one other fact of importance: it contains (apart from the amylase that does

not here concern us) not only a trypsin, but also an erepsin. This is made probable by the fact that at neutral reaction the extract will digest peptone with rapidity, but is able to make very little impression upon native albumin. The glycerin extract contains but traces of protein. With three parts of alcohol but a slight yellowish precipitate is produced. Tannic acid, however, brings down a much heavier precipitate.

It is my intention to repeat these experiments next season upon a very large scale, with the use of fresh ferments from different sources, and with the experiments extended over longer intervals of time, up to a year at least. With proper technique, it ought to be possible, without violating the conditions of bacterial sterility in the tests to introduce at regular intervals fresh portions of active ferments, in order that the enzymic action may be maintained at the maximum, and the inactivation of the ferments circumvented. So far as I can determine by comparative tests, the trypsin from the clam differs from the trypsin of higher mammals only in an increased resistance to hydrolysis; in general the ferments from the cold-blooded animals, especially the marine animals, are much more resistant than those derived from higher animals. If the fresh pancreatic juice of the dog were added each week to such a reversion experiment, the result ought to be the same. In time I shall have such experiments to report. Some of the ferments of vegetable origin deserve also a trial.

Some of the analyses herein reported were done in the laboratory for physical chemistry of the Nobel Institute, Stockholm, and in the chemical laboratory of the Stockholm High School. For these facilities, I take pleasure in expressing my appreciation to Prof. Svante Arrhenius, and to Prof. H. v. Euler.



# ON THE COMPOSITION AND DERIVATION OF PROTAMIN.

By ALONZO ENGLEBERT TAYLOR.

(From the Hearst Laboratory of Pathology, University of California.)

(Received for publication, December 12, 1908.)

According to the investigations of Kossel and Dakin<sup>1</sup> the protamin of the salmon yields on hydrolysis arginin, an amino-valerianic acid, serin and prolin. Abderhalden<sup>2</sup> analyzed a preparation of salmin and reported further the positive finding of leucin, alanin, with probably phenylalanin and aspartic acid. Having in hand material of exceptional purity, I have been able to reinvestigate the matter, and here report the results of this study, together with some general considerations bearing upon the question of the derivation of protamin in the metabolism of the salmon. The results of my analyses show that the statements of Kossel and Dakin are correct, those of Abderhalden in error.

To obtain a pure protamin, much more is necessary than to follow the methods of Miescher. The desiderata are of two kinds: the proper collection of the raw material, and the methods of Kossel for the isolation of the base. The ripening of the salmon testicle is a process lasting weeks. Careful studies upon the part of those engaged in the extensive salmon hatcheries in California have shown that from four to six weeks are consumed in the ascent of the fish to the mouths of the Battle Creek and the McCloud river, where the hatcheries are situated. Salmon caught in the brackish water of the bays of San Francisco and San Pablo and in the tide waters of the lower Sacramento river (where the fish still feed), display in their testicles not a little protamin. Some of the male fish are ripe on their

<sup>1</sup> Kossel and Dakin: *Zeitschr. f. physiol. Chem.*, xl, pp. 311, 565, 1904; xli, p. 407, 1904.

<sup>2</sup> Abderhalden: *Zeitschr. f. physiol. Chem.*, xli, p. 55, 1904.



arrival at the waters of the hatcheries; others remain for weeks after their arrival in the impounding basin before the sperm has ripened. The process of ripening has two main stages: the maturation of the spermatazoa, which requires weeks, probably months; and the liquefaction of the intercellular connective tissue of the testicle, whereby the testicular tissue becomes converted into fluid sperm. This process of liquefaction does not occur in the testicle *en bloc*, but extends through some ten days; in other words, the entire testicle does not become ripe at once. In the practical operation of the hatchery utilization is made of this fact. As the salmon ascend the stream on which the hatchery is located, they are trapped and impounded in a large basin in the stream, salmon-runs being installed at the lower end of the pond, while above the further ascent of the fish is barred with wooden gratings. This pond is seined several times daily. All the "ripe females" caught are retained and placed in crates; the "unripe females" are returned to the pond. Only so many of the "ripe males" caught are retained as may be necessary to keep the pens designed for them filled, the rest are returned to the pond. For the purpose of the hatchery the males are always in large excess. At a certain time of each day, the "ripe females" caught upon the previous day are killed and their eggs placed in containers. The male salmon are taken from their pens, allowed to lie upon a platform out of water until they are so far stupefied by lack of respiration that they may be handled, when they are held over the dishes containing the eggs and the sperm ejected therein by gently milking the ventral surface of the fish. When no further flow of sperm is to be obtained, the male fish are returned to their pens, where recovery soon occurs. The male fish will be stripped in this manner daily until the entire testicle is liquefied and the fish exhausted. A healthy male salmon can be stripped daily for from six to ten days. Protamin prepared from unripe testicles will be mixed with protein, histone, and amido-acid in amounts proportional to the immaturity of the material, due to the fact that the unripe glands contain blood, tissue protein, connective tissue and histone. Protamin prepared from ripe glands will contain but traces of these, since no blood, cellular protein or histone remain in the glands, while there will be traces of nucleinic

acid, and of amido-acids derived from the liquefaction of the intercellular connective tissue. The nucleinic acid and the amido-acids tend to cling to the protamin sulphate when the latter is precipitated with alcohol from aqueous solution. If the salmin sulphate be separated from a hot saturated solution in the oil-like state, most of the salts and a part of the organic impurities, especially the proteins and histone, will be removed. If later the combination of the picrate be prepared, the remainder of the organic impurities will be removed if the initial material was mature. The proteins cannot be entirely removed in this way. If they be present in considerable amount they will pass into the oil-like state in a condition of adsorption, and will remain with the protamin when precipitated with picric acid; when the protamin is finally recovered, part of the protein will be found with it. If one is working with a protamin sulphate obtained from unripe testicles, where the admixture of protein and histone is large, one carrying-through of these two manipulations is not sufficient to insure a pure product, as the protein clings tenaciously; if one is working with a fully ripened material, containing the least traces of protein, one will secure a pure product, as the nucleinic acid and amido-acids will be completely removed by the two procedures of purification. In other words, the method of Kossel is adapted to yield a pure product when applied to a ripened initial material, for which it was in fact devised; it is not suited to the manipulation of unripe initial material, nor is any known method. These facts I have stated in detail, because to judge from the work of Abderhalden, they do not seem to be generally known. In his publication<sup>1</sup> he says: "Den Einwand, den man übrigens gegen jede Untersuchung an Proteinstoffen erheben kann, dass das untersuchte Material keine Garantie für chemische Einheitlichkeit biete, suchte ich durch möglichst sorgfältige Darstellung einzuschränken." He does not state in what the special precautions in the preparation consisted. In his later text-book,<sup>2</sup> following the appearance of the article of Kossel and Dakin, he states in reference to this preparation of salmin, that his own results \* \* \*

<sup>1</sup> Abderhalden: *loc. cit.*, p. 56.

<sup>2</sup> Abderhalden: *Lehrbuch der physiologischen Chemie*, pp. 191, 192, 1906.

"welcher von einem sorgfältig gereinigten Salmin zur Beobachtung kam, dürfte vielleicht in der nicht völligen Reife der Hoden, aus denen das betreffende Produkt gewonnen war, seine Erklärung finden." It is to be inferred that Abderhalden obtained his material from a distance and at second hand, and was not therefore in a position to control the collection of the material.

Sixty grams (calculated as the base) of most carefully purified protamin from the quinnat salmon of the Pacific coast, obtained from fully matured material, were hydrolyzed with hydrochloric acid and the resultant solution of amido-acids submitted to the Fischer ester method. In the distillation of the esters but two fractions were made: the first contained the esters that would distill over at less than  $100^{\circ}$  on a water bath at a pressure of 10 mm.; the second fraction contained all that would distill over up to  $200^{\circ}$  on an oil bath, at a pressure of 10 mm. The first fraction was saponified by prolonged boiling in water, the resultant solution carefully concentrated to a syrup, and this then dried to a gummy mass in a stream of hot air at  $50^{\circ}$ . It was then extracted with warm absolute alcohol three times. The combined alcoholic extracts were evaporated to dryness and the residue again extracted with absolute alcohol, this time with a large volume, over night at room temperature. A slight residue remained which was returned to the original residue of the alcoholic extraction. The amido-acid contained in the alcoholic extract, presumably prolin, was secured by crystallization on the evaporation of the alcohol. It weighed 6.5 grams. A portion of this was twice recrystallized from absolute alcohol, and submitted to analyses for carbon, hydrogen and nitrogen, the latter by the Kjeldahl method.

The results were:

	Calculated for $C_8H_9NO_2$ :		Found:
C.....	52.2	per cent.	52.0 per cent.
H.....	7.8	"	8.0 "
N.....	12.2	"	12.1 "

The figures agree well with those for prolin.

The residue after the extraction with alcohol was dissolved in the minimum amount of hot water, filtered to remove a slight flocculation, and hot alcohol added until the first traces of cloudi-

ness appeared. On cooling and standing over night, a heavy crystallization occurred. The crystals were collected by filtration and the filtrate evaporated to dryness; but a slight trace of a yellowish gummy material remained, weighing but a few milligrams. The crystals obviously included the entire solute. These crystals were again dissolved in hot water and hot alcohol added until cloudiness appeared. On cooling and standing over night, the crystals had again separated out. They were collected, washed with dilute, then absolute alcohol, finally with ether; the weight was 3.21 grams. The crystals were twice recrystallized from alcohol. On analysis the substance, presumably amidovalerianic acid, gave the following figures:

	Calculated for $C_8H_{11}NO_2$ :	Found:
C.....	51.3 per cent.	51.1 per cent.
H.....	9.4 "	9.5 "
N.....	11.9 "	12.1 "

The figures agree well with those for amidovalerianic acid.

The fraction of ester secured at the higher temperature was saponified by heating with baryta water, the barium carefully removed with sulphuric acid, filtered hot, the filtrate concentrated to a syrup, to which hot alcohol was added until a cloudiness appeared. On cooling and standing over several days a heavy crystallization appeared. The crystals were filtered off, and the filtrate evaporated to dryness; but a trace of gummy material remained. The crystals again obviously represented the entire solute. The crystals were three times recrystallized from water-alcohol, and weighed 5.22 grams. The substance, presumably serin, gave on analysis the following figures:

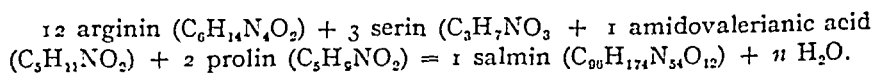
	Calculated for $C_3H_7NO_3$ :	Found:
C.....	34.3 per cent.	34.2 per cent.
H.....	6.6 "	6.8 "
N.....	13.3 "	13.5 "

The figures agree well with those for serin. In all these figures I have used the rounded figure for the first decimal, since it can be easily shown that the second decimal has no certainty in an elementary analysis.

Further identification of these substances I have not carried out, on account of enforced absence from the laboratory. Were the subject new, further identification in the qualitative sense would be necessary; in view of the work of Kossel and Dakin, however, I do not consider that it can be doubted that the substances of the denominated percentage compositions were prolin, serin and amidovalerianic acid. It is also clear from the results of the evaporation of the filtrates that the three amido-acids are all the monamido-acids that were obtained from the salmin by the Fischer ester method.

A further lot of salmin sulphate (30 grams calculated as salmin) was hydrolyzed with hydrochloric acid and the arginin isolated according to the method of Kossel. The amount was 27.52 grams. From all the figures it is evident that for the preparation of salmin here under analysis:

One hundred parts protamin + 11 water = 91.73 parts arginin + 10.83 parts prolin + 5.35 parts amidovalerianic acid + 8.70 parts serin. Kossel and Dakin<sup>1</sup> have attempted, from the quantitative data of a similar analysis, to determine the composition and molecular weight of salmin. They suggested two formulæ: 10 mol. arginin, 2 mol. serin, 1 mol. amidovalerianic acid, and 2 mol. prolin, leading to the formula,  $C_{81}H_{155}N_{45}O_{18}$ ; and 12 mol. arginin, 2 mol. serin, 1 mol. amidovalerianic acid and 3 mol. prolin leading to the formula,  $C_{98}H_{180}N_{54}O_{21}$ . Neither of these formulæ gives the carbon:nitrogen ratio observed in the analyzed preparations of salmin— $C_{10}H_{20}N_9O_2$  and  $C_{30}H_{50}N_{17}O_7$ . The later analyses by Goto of the salts with platinum chloride agree exactly with neither of these. An equation that does give exactly the carbon:nitrogen ratio of the first of the above two equations, and which also, with one exception, presents a good agreement with the analytical results herein reported is the following:



Comparing now the amounts of arginin, serin, prolin and amidovalerianic acid which such a molecule would yield on hydrolysis with the figures for the amounts obtained in the analyses herein reported, we have in 100 parts:

<sup>1</sup> Kossel and Dakin: *loc. cit.*, xli, p. 414, 1904.

	Calculated for $C_{96}H_{174}N_{54}O_{12}$ :	Found:
Arginin.....	94.07	91.73
Prolin.....	10.16	10.83
Valin.....	5.46	5.35
Serin.....	13.91	8.70

The agreement in the case of arginin, valin and prolin is good, in the case of serin very poor. It is, however, known that serin is isolated with great difficulty, being apparently in the state of the ester not well extracted with ether, and it is possible to account for the deficit in some such way. Upon the basis of this equation, the molecular weight of salmin is 2274, a figure vastly lower than those known approximately for the higher forms of protein. In his earlier investigation Kossel was led to define the protamins as the simplest forms of protein. Their general behavior was so different from that of the common forms of protein, that at that time objections were energetically raised against the application of the term protein to protamin. The studies of the Fischer school upon the amido-acids contained in the proteins have fully established the correctness of Kossel's position that protamin is as typical a protein as serum albumin. Most of the higher forms of protein contain from ten to fifteen different amido-acids while salmin contains but four. A consideration of the relations of spacial isomerism as applied to the linkings of the amido-acids in a molecule of protein will indicate that a substance containing four such components is certain to be more simple in its chemical structure than one containing a dozen components. The figures given above have naturally only a provisional value, though from the theoretical point of view, an undoubted importance does attach to them.

Kossel<sup>1</sup> has sketched an interesting scheme for the derivation of the protamins. Protamin he regards as being derived from histone, and this to have been built up in the salmon from the fragments (amido-acids) of the disintegration of protein of muscle since the testicle is in part formed during the period during which the salmon ascend the streams to spawn, at which time they fast and their muscles waste; in the case of the other fish, the histones are naturally to be derived from the amido-acids (respt. protein)

<sup>1</sup> Kossel: *Zeitschr. f. physiol. Chem.*, xliv, p. 347, 1905.

of the diet. This general scheme, which has in its favor every known fact, can in the opinion of the writer be made much more definable if it be interpreted from the standpoint of reversible reactions. The facts are few and certain. In the testicle of the salmon of the average size are formed some 30 grams of protamin. To provide the necessary amido-acids, less than half a kilo of muscle would be required. The migrating salmon does not feed after leaving salt or brackish water. At this time the testicles are rich in histone and contain not a little protamin, derivable from the proteins of the diet; the amount that must be later formed during the period of fasting is thus much less than the total, and the figure of a half kilo of muscle is largely excessive. The observations of the experts in charge of our fish hatcheries tend to show that the male salmon lose from 2 to 5 kilos in weight during the period of migration and spawning. This is partly fat, partly muscle. During this period the fish are actively engaged in ascending streams against the current, in driving marauders from the nests and in combats with each other, all of which require maintenance, in part without question by muscular tissue. Nevertheless, it is certain that the amido-acids required for the formation of the histone are easily available in the muscle known to be disintegrated during this period, and there is no necessity to consider any other source. The hypothetical question concerns the *modus operandi* of the formation of histone from the amido-acids of the muscle.

When proteins are ingested, they reappear in the circulation in the form of serum albumin and globulins. No matter what the protein in the diet, in the stage of resorption there is an intramolecular rearrangement, so that the circulating protein is in the state of serum albumin and globulins. These blood proteins are carried in the circulation to the various tissues, and from them the cells form myosin, reticulin, gelatin, etc. These transformations concern not only intramolecular rearrangements, with the maintenance of the same molecular mass; but include also the introduction or extrusion of particular groups of amido-acids. In general, the common proteins contain the same amido-acids, but in different amounts and inferentially in different linkings; but some proteins contain certain amido-acids, such as cystin or phenyl-amido-acid, that are not present in others, or but to

slight degree. The faculty of the body to effect such intramolecular rearrangements is well illustrated in the sugar metabolism. No matter in what form sugar is ingested, in the blood we find but one form, *d*-glucose; in other words, the levulose and galactose are converted into *d*-glucose by intramolecular rearrangement. This glucose in the blood passes into the active mammary gland, where it is in part reconverted into *d*-galactose, which combined with *d*-glucose forms milk sugar. *D*-galactose is also formed from *d*-glucose in the central nervous system. That the different hexoses tend thus to pass into each other was first shown by Lobry du Bruyn and van Erenstein;<sup>1</sup> it has been experimentally demonstrated for the mannoses by Neuberg and Mayer.<sup>2</sup> In this same manner we assume that the protein of muscle is formed from the proteins of the blood serum in the salmon. For the formation of the histone of the testicle in the salmon, we may now choose one of two conceptions. According to the one apparently adopted by Kossel, the muscle protein is disintegrated *in situ*, and the amido-acids thus set free in the circulation are carried to the testicle, there to be converted into histone. The second possible conception is to assume that the protein of the blood serum is taken into the testicular tissue, there hydrolyzed by enzymic action, and the histone then built up from the amido-acids. The reduction in the proteins of the blood serum thus occasioned is then made good by the reversion of muscle protein into serum protein, the reversal of the reaction whereby during the period of feeding the muscle protein is formed from the serum protein. In the same manner, as the protein of the blood serum is burned to maintain the movements of the fish, the loss is made good by the reversion of muscle protein into serum protein. Just as the blood furnishes *d*-glucose to the mammary gland, which forms from it *d*-galactose, so the blood furnishes to the testicle of the fasting salmon the common protein, from which histone is formed. In the hypothesis of Kossel, the histone is formed from amido-acids derived from the cleavage of muscle protein in the muscles and transported to the testicle by

<sup>1</sup> Lobry de Bruyn and van Erenstein: *Ber. d. deutsch. chem. Gessellsch.*, xxviii, pp. 3078, 3085, 1898; *Rev. Chim. d. Pays-Bas.*, xiv, pp. 116, 203, 1898; xvi, p. 263.

<sup>2</sup> Neuberg and Mayer: *Zeitschr. f. physiol. Chem.*, xxxii, p. 256, 1901.



the circulation. In the hypothesis here suggested, the histone is formed from the proteins of the blood serum, the cleavage of which is assumed to occur in the testicle; since the blood proteins are in the fasting fish held to be derived from the muscle protein the histone is of course derived from the muscle, though indirectly. In favor of the hypothesis here suggested is the fact that the proteins of the blood serum constitute the substratum from which are formed all the specialized proteins of the body, and it is natural to believe that this modus applies also to the formation of histone. In this entire argument it is assumed that histone is constructed from preformed amido-acids; that these might be synthesized in the testicle is not impossible, but there is every reason to believe that the building-stones of the histone, and thus of protamin, are derived from the hydrolysis of the body protein.

# ON THE QUESTION OF THE IDENTITY OF PEPSIN AND CHYMOSIN.

By ALONZO ENGLEBERT TAYLOR.

(From the Hearst Laboratory of Pathology, University of California.)

(Received for publication, December 12, 1908.)

Since the earliest days in the study of the digestive ferments, pepsin and chymosin have been considered as distinct substances endowed with properties of very different nature, though both the secretion-products of one organ. Within recent years, however, through the work of Nencki and Sieber,<sup>1</sup> Schoumow-Simanowski,<sup>2</sup> Sawjalow,<sup>3</sup> Gewin,<sup>4</sup> and especially of Pawlow,<sup>5</sup> the idea has been advanced that they are in reality but one substance. The older conception has been defended by Schmidt-Nielsen,<sup>6</sup> Bang,<sup>7</sup> Hemmeter,<sup>8</sup> Jacoby<sup>9</sup> and especially by Hammarsten,<sup>10</sup> to whose work so much of our present knowledge of the coagulation of milk is due. Owing to the indefiniteness of our conceptions of the chemical nature of the coagulation of milk, the proponents of the unistic theory cannot be expected to put into concrete terms just what they conceive the action of pepsin upon the milk to be, but in general terms we may say that the conversion of casein into paracasein is held to be the result of the proteolytic action of the pepsin upon the casein (probably a reaction of hydrolysis), the actual coagulation of the paracasein being, as

<sup>1</sup> Nencki and Sieber: *Zeitschr. f. physiol. Chem.*, xxiii, p. 291, 1901.

<sup>2</sup> Schoumow-Simanowski: *Arch. f. exper. Path. u. Pharm.*, xxxiii, p. 336, 1899.

<sup>3</sup> Sawjalow: *Zeitschr. f. physiol. Chem.*, xlvi, p. 307, 1905.

<sup>4</sup> Gewin: *Ibid.*, liv, p. 32, 1907.

<sup>5</sup> Pawlow and Parastschuk: *Ibid.*, xlii, p. 415, 1904.

<sup>6</sup> Schmidt-Nielsen: *Ibid.*, xlviii, p. 92, 1906.

<sup>7</sup> Bang: *Ibid.*, xliii, p. 358, 1904.

<sup>8</sup> Hemmeter: *Berl. klin. Wochenschr.*, Ewald Festnummer, 44, 1905.

<sup>9</sup> Jacoby: *Biochem. Zeitschr.*, i, p. 53, 1905.

<sup>10</sup> Hammarsten: *Zeitschr. f. physiol. Chem.*, lvi, p. 18, 1908.

generally admitted, a purely secondary and extraneous process. The unistic conception is with strict consistency applied to the pancreatic juice, which contains also a curdling ferment; this is held to be identical with the trypsin. The proposition of the unistic school is in chemical terms to be stated concretely as follows: the coagulating and the proteolytic properties of the molecule of pepsin reside in different groups within the molecule, just as an organic molecule may contain a carboxyl and an hydroxyl group. There is unquestionably a legitimate application of the well-known facts of organic chemistry to this problem. In innumerable instances it is known that reactions of different types may be carried on independently with the different groups contained in a large organic molecule or even in relatively small ones. Upon the basis of the current conception of fermentative acceleration as consisting in the establishment of intermediary reactions, the chemical properties of the different groups within the molecule would determine whether a certain substance could be an accelerator for a certain reaction; and a single molecule could in all correctness be assumed to contain different groups that would qualify it to act as the accelerator for different reactions. The digestion of protein is an act of hydrolysis; the presence of pepsin introduces into the reaction between the protein and water new intermediary steps whereby the internal resistance in the molecule of pepsin is reduced and the velocity of the cleavage increased. There is no theoretical reason why the molecule of pepsin should not contain another group that bears the same relation to the reaction between casein and water, whatever the nature of that reaction may be. A simple scheme will make the matter clear.

#### AR-PEPSIN-RB

Protein + water = Proteose (slow reaction).

Protein + water + AR-Pepsin-RB = Proteose (rapid reaction).

Casein + water = Paracasein (slow reaction).

Casein + water + BR-Pepsin-RA = Paracasein (rapid reaction).

There can be no theoretical reason why the two reactions should not be carried on simultaneously. Whether this scheme is true or not, is a matter to be determined by investigation. Whether the assumption be a natural and obvious one or a distant and

vague conjecture, is also of no consequence so far as the theory of fermentation is concerned; upon the theory of fermentation as representing the acceleration of a reaction through the reduction in the internal resistance by the installation of new intermediary reactions, the hypothesis of Pawlow is logical and permissible. It is possible to go further; there is no reason why all the enzymic activities of the pancreatic juice (lipolytic, proteolytic, amylolytic, inversion and coagulation) should not be ascribed to the different groups of one organic molecule. It is not necessary to invoke the Ehrlich hypothesis; the theory of catalytic reactions and the facts of organic chemistry are broad enough to support the proposition. For the future investigation of the question it will be necessary to determine in every way the relations of coagulation and proteolysis.

Gastric secretions occur in man, under conditions of disease, in which the proteolytic property remains and the coagulating property is absent. It has been long known by clinicians that in individuals suffering from carcinoma of the stomach, the disappearance of chymosin from the gastric contents was apt to occur early, and a certain diagnostic value has been attached to this finding. I have observed, in the examination of many gastric contents from cases of pyloric carcinoma, five instances in which the gastric contents would digest protein but could not coagulate milk. In order to determine the actual state of secretion in the subjects of this disease, certain preliminary procedures are necessary. The stomach should be carefully cleaned by lavage on several successive days, and the diet carefully regulated. Then the patient is to be given an Ewald test-meal and a dose of hydrochloric acid, say 10 cc., well diluted. After an hour and a half the gastric contents are removed by expression, and the tests for the different secretions carried out. In the cases here reported, the proteolytic activity was tested upon coagulated egg-albumin. The coagulating activity was tested by adding the neutralized juice to fresh neutral milk in regularly varying amounts, and the tubes held in the thermostat; for controls plain milk and milk to which the neutralized boiled gastric contents had been added, were employed. In five instances in my experience, the gastric juice displayed proteolytic activity, but would not coagulate milk.

Such facts would be interpreted directly and naturally by Hammarsten as follows: Pepsin is one substance, chymosin is another substance; in these cases of carcinoma of the stomach, the secretion of the one substance, pepsin, is not affected, the secretion of the other substance, chymosin, is inhibited. According to Pawlow, the interpretation would be something as follows: Pepsin contains two reaction-groups, one accelerating the hydrolysis of protein, the other accelerating the conversion of casein into paracasein; in these cases of carcinoma of the stomach, this substance pepsin has been so modified in the cells in which it was formed or after its formation there, that the group in which the proteolytic function resides has remained unaltered, while the group in which resides the coagulating property has suffered such alteration as to deprive it of its reaction qualities. A decision of the question, such facts as those here recorded cannot yield.

In favor of the unistic interpretation is the parallelism that is usually found in gastric contents between the proteolytic and the coagulating activities. Against the unistic theory are the following facts; it is possible by thermic and chemical manipulation to affect the one activity and not the other. It is possible to prepare pepsin, free from chymosin, and chymosin, free from pepsin. Jacoby<sup>1</sup> prepared an antiserum by the injection of gastric ferments into animals; the antiserum contained an anti-chymosin, but no anti-pepsin. In the case of the pancreatic juice, Vernon<sup>2</sup> and Wohlgemuth<sup>3</sup> have shown that the action and the activation of the trypsin and the coagulating ferment run parallel. The antiserum obtained by Jacoby after injections with pancreatic juice was both anti-tryptic and anti-coagulating.

To the students of ferments it must seem that the data at present at hand are best interpreted as indicating that pepsin and chymosin are different substances. This provisional conclusion can be combated by showing that in all the chymosin-free pepsins and pepsin-free chymosins the inactivity of the one function is the result of inhibiting substances (as was shown for Glässner's chymosin-free pepsin by Pekelharing<sup>4</sup>), and by the

<sup>1</sup> Jacoby: *loc. cit.*

<sup>2</sup> Vernon: *Journ. of Physiol.*, xxix, p. 302, 1903.

<sup>3</sup> Wohlgemuth: *Biochem. Zeitschr.*, ii, p. 350, 1907.

<sup>4</sup> Pekelharing: *Arch. d. sci. biol.*, xi, suppl., p. 36, 1906.

demonstration that the coagulating activity can be restored to chymosin-free pepsin and the proteolytic activity to pepsin-free chymosin. Naturally a chemical group can be so completely removed by substitution that its reaction-qualities cannot be restored; but this must be demonstrated to occur in the preparation of pepsin-free chymosin and chymosin-free pepsin, unless the above stated requirements can be fulfilled. The theory that pepsin and chymosin are one substance cannot be proved by quoting the lateral chain hypothesis of Ehrlich; the application of this hypothesis to the phenomena of fermentation in the present state of our knowledge is arbitrary and devoid of experimental basis.



## ON THE INVERSION OF CANE SUGAR AND MALTOSE BY FERMENTS.

BY ALONZO ENGLEBERT TAYLOR.

*(From the Hearst Laboratory of Pathology, University of California.)*

*(Received for publication, December 12, 1908.)*

The recent papers of Hudson<sup>1</sup> induce me to publish some figures that I have obtained in work upon the same subject, but which I have for some time held back pending a larger investigation of the subject. In my work I obtained the same results as those obtained years ago by O'Sullivan and Thompson for cane sugar; in the individual series, the rate of inversion was proportional to the concentration of the substrate, and when the values were calculated according to the equation for a monomolecular reaction, constants in good agreement were obtained. With different initial concentrations of the substrate, however, all other conditions being identical, the constants in the different series did not agree. The results have been held back pending the investigation of this lack of agreement in series of different initial concentrations. Since the publication of the results of the investigations of Hudson, which have confirmed the results of O'Sullivan and Thompson, there is no longer any purpose in withholding the figures, pending the study of the unsolved question now being undertaken by Professor Arrhenius and myself. In my work with cane sugar and maltose, I followed the methods of O'Sullivan and Thompson, the solutions were made alkaline before being placed in the polariscope. For maltase I employed a taka-diastrase, which is rich in maltose; for sacchrase, I used a preparation from brewer's yeast. I have always used low concentrations. Temperature 140°. As illustrations I give a series each for maltose and saccharose, the con-

<sup>1</sup> *Journ. of the Amer. Chem. Soc.*, xxx, pp. 1160 and 1564, 1908.



stants being calculated according to the equation for a monomolecular reaction:  $\frac{dx}{dt} = C(A-x)$ .  $t$  are hours.

Saccharose: concentrations  $\frac{1}{2}$ , 1 and 2 per cent. Ferment concentration constant in all. Constants are  $\times 10^{-4}$ .

(t)	1	2	3	4	5	6	7	Mean.
<i>per cent</i>								
$\frac{1}{2}$	420	410	416	430	426	428	434	423
1	320	330	312	322	338	332	326	326
2	160	165	160	172	176	168	170	167

Maltose: concentrations 1, 2 and 3 per cent. Ferment concentration constant. Constants are  $\times 10^{-5}$ .

(t)	1	2	3	4	5	6	7	Mean.
<i>per cent</i>								
1	360	362	368	370	376	348	354	363
2	220	268	222	242	238	242	238	239
3	140	132	136	160	140	138	145	142

To these I will add, from work already published, the constants for a similar experiment in the digestion of starch with salivary amylase. The substrate concentrations were  $\frac{1}{4}$ ,  $\frac{1}{2}$  and  $\frac{3}{4}$  per cent. Time is minutes. The temperature was  $35^{\circ}$ . The constants are  $\times 10^{-6}$ .

(t)	30	45	60	75	90	120	150	180	Mean.
<i>per cent</i>									
$\frac{1}{4}$	490	465	455	470	465	455	560	455	477
$\frac{1}{2}$	430	420	390	415	405	395	430	410	412
$\frac{3}{4}$	390	370	385	390	480	370	365	370	390

In all these series it is clear that the conformity in the constants in each series is satisfactory. Between the constants in the several series, however, the conformity is not present. On the contrary the lower the substrate concentration the higher the figure for the constant. This behavior of the figures for the con-

stants is to be seen in the results of many investigators in this field, though the matter has been given little discussion. In the experiments whose constants are given above, the total conversions were about 60 per cent for the lower concentrations, less of course for the higher concentrations. The behavior of the constants does not lie in this, as it persists if the conversions be carried on so that the several series overlap. Under the strict application of the law, the constants should be the same for the tests with  $\frac{1}{2}$ , 1 and 2 per cent substrate concentrations. In fact, they are not, though they are in conformity in the individual series. This state of affairs is, however, not peculiar to the reaction of fermentation, but is seen, though to a less pronounced extent, in other catalytic reactions. For example, the constants for the acid inversion of cane sugar are not identical in 10 and 30 per cent substrate concentrations; the difference here observed is, however, much less than is observed in the case of the action of invertase, and is in any event proportional to the osmotic pressure.

My experiments, therefore, confirm the results recently published by Hudson for cane sugar—that the fermentation follows the course of a monomolecular reaction—and indicates that the same holds true for the fermentation of maltose. In this last, my results are contradictory to those recently published by Mlle. Piloche,<sup>1</sup> according to which the constants are regular only when calculated according to the equation of Henri. A careful review and recalculation of the data in her paper, however, show that the results are not regular; in some experiments the normal law is followed, and in other cases there is a maximum in the curve, the cause of which cannot be discerned from the publication and will need to be determined and elucidated by additional experimental work.

<sup>1</sup> Piloche: *Journal de chimie physique*, vi, p. 229, 1908.



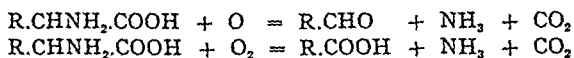
# NOTE ON THE OXIDATION OF GLUTAMIC AND ASPARTIC ACIDS BY MEANS OF HYDROGEN PEROXIDE.

By H. D. DAKIN.

(From the Laboratory of Dr. C. A. Herter, New York.)

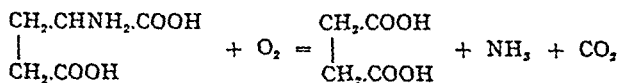
(Received for publication, December 21, 1908.)

In previous communications<sup>1</sup> it has been shown that a number of amino-acids of the typical formula  $R.CH.NH_2.COOH$ , undergo oxidation with hydrogen peroxide so as to yield ammonia, carbon dioxide and an aldehyde  $R.CHO$ , the latter being more or less oxidized to the corresponding acid, according to the conditions of the experiment:



This reaction was found to occur in the case of glycocoll, alanin,  $\alpha$ -amidovaleric acid,  $\alpha$ -amido-isovaleric acid, leucin and phenylalanin, and was of especial interest on account of the similarity of the products of oxidation with those obtained through some biochemical oxidative changes.

The following experiments were undertaken in order to ascertain whether the reaction could be extended to the dibasic amino-acids. This was found to be the case. Glutamic acid proved to be readily oxidized when the ammonium salt was gently warmed with hydrogen peroxide, with liberation of ammonia and carbon dioxide and formation of large quantities of succinic acid. The reaction may be expressed as follows:



<sup>1</sup> This *Journal*, i, pp. 171 and 271; iv, p. 63.

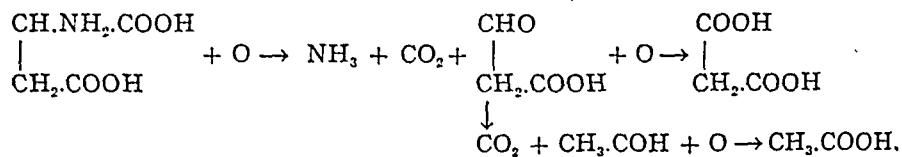
The reaction is clearly analogous to the oxidation of the mono-amino-acids under similar conditions. Aldehyde-butyric acid,<sup>1</sup>  $\text{COH} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{COOH}$ , may be considered as an intermediary product, but no indications of the presence of this substance were obtained.

The formation of succinic acid from glutamic acid through oxidation with hydrogen peroxide is of interest since a precisely similar reaction is brought about by the action of yeast (Ehrlich) and also through the agency of putrefactive organisms.<sup>2</sup>

The oxidation of aspartic acid with hydrogen peroxide showed that essentially the same reaction took place. The change is complicated, however, by the fact that the primary product of the reaction, namely the half aldehyde of malonic acid is very unstable and breaks up with extreme ease into acetaldehyde and carbon dioxide, both of which products were readily identified. A small amount of malonic acid was isolated, however, it doubtless being formed from the further oxidation of the aldehyde acid.

In addition, minute quantities of acetic acid and formic acid resulting from the further oxidation of the acetaldehyde were identified.

The changes may be represented as follows:



#### EXPERIMENTAL.

*Oxidation of glutamic acid.* One-tenth gram mol. of glutamic acid was neutralized by the addition of a slight excess of ammonia and then gently warmed to about  $70^\circ$  with 0.3 gram mol. of 2.5 per cent hydrogen peroxide. After about an hour the liquid was distilled. The residue did not reduce Fehling's solution, aldehyde-butyric acid was therefore absent. It was acidified with phosphoric acid and extracted with ether in a continuous

<sup>1</sup> Perkin and Sprankling: *Trans. Chem. Soc.*, lxxv, p. 16.

<sup>2</sup> W. Brasch and C. Neuberg: *Biochem. Zeitschr.*, xiii, p. 299.

extraction apparatus. On distilling off the ether a crystalline residue of succinic acid was obtained amounting to 47 per cent of the theoretical amount. It was recrystallized from water and melted at  $180-181^{\circ}$ . A considerable amount of unoxidized glutamic acid was present in the solution after extraction with ether, so that by repeating the oxidation it would be possible to still further improve the yield of succinic acid.

*Oxidation of aspartic acid.* Sodium aspartate, 0.1 gram mol., was oxidized with 0.3 gram mol. of neutral hydrogen peroxide. A trace of ferrous sulphate was added as a catalyst. After standing for a few minutes at the ordinary temperature of the room, the liquid became warm and a vigorous reaction took place with evolution of much carbon dioxide. The liquid was acidified and distilled. The distillate contained acetic and formic acids equivalent to approximately 0.25 gram of acetic acid. In addition all the usual reactions for acetaldehyde were obtained including Rimini's reaction with sodium nitroprusside and piperidine. The distillate was redistilled and the first portion to come over was treated with para-nitrophenylhydrazine acetate in the usual way. The small amount of precipitated hydrazone was recrystallized from alcohol and melted at  $127-128^{\circ}$ . The para-nitrophenylhydrazone of acetaldehyde melts at  $128-128.5^{\circ}$ .

The residue from the first distillation was acidified with phosphoric acid and extracted with ether. On evaporation of the ether a crystalline residue of malonic acid was obtained. It was identified by its melting point ( $131-132^{\circ}$ ) and by its rapid decomposition with liberation of carbon dioxide at the same temperature. The yield of malonic acid was considerably less than the corresponding yield of succinic acid from the oxidation of glutamic acid.



## THE ACTION OF GLYCOCOLL AS A DETOXICATING AGENT.<sup>1</sup>

By H. D. DAKIN.

*(From the Laboratory of Dr. C. A. Herter, New York City.)*

(Received for publication, December 21, 1908.)

The power of the living organism to convert poisonous substances into easily soluble, relatively non-toxic, compounds which are excreted by the kidneys is commonly spoken of as a protective mechanism. The detoxication of the poisonous substance is commonly effected through oxidation, reduction, hydrolysis or union with some other substance such as glycocoll, sulphuric acid, glucuronic acid, etc., or a combination of these processes. But the fact that a substance when introduced into the body undergoes one or the other of the reactions just mentioned is not in itself sufficient proof that the original substance was toxic, although this appears not infrequently to be tacitly assumed. Thus, for example, benzoic acid when introduced into the body is promptly converted into hippuric acid and eliminated by the kidneys, but it is by no means clear that this is an actual protective mechanism for we know nothing of the action of benzoic acid itself upon the animal organism. By administering very large doses of benzoic acid to some animals, it is true that a small portion of it may be excreted unchanged, but observations under these conditions when the experiment is complicated by the simultaneous formation and excretion of large amounts of hippuric acid can teach us but little. As regards the lower organisms such as bacteria, Dr. Herter informs me that he has observed but little difference in the toxicity of equivalent sodium benzoate and sodium hippurate. It is clear therefore that the coupling of a substance with glycocoll in its passage through the organism

<sup>1</sup> The term "detoxicating agent" is used in this paper as an equivalent of the widely used German word "Schutzmittel."



is in many cases not definitely proven to be a protective mechanism.

The present paper contains definite examples of the lowering of the toxicity of an aromatic acid resulting from its combination with glycocoll and also contains an account of experiments upon the relative ease of oxidation of three aromatic acids and of the products formed by their union with glycocoll. These experiments show that the resistance of the glycocoll derivatives to oxidation is enormously greater than that of the free unpaired acids. It might be thought that union with an amino-acid such as glycocoll might bring the substance more readily within the sphere of the oxidizing ferments of the cell, for it is known that the amino-acids are readily withdrawn from the general circulation. This appears, however, not to be the case.

The substances examined were as follows:

	Substance.	Formula.	Result.
I	Phenylpropionic acid . . . . .	$C_6H_5.CH_2.CH_2.COOH$ . . . . .	Toxic, lethal dose = about 0.8 gm. per kilo. Easily oxidized.
	Phenylpropionyl-glycocoll . . . .	$C_6H_5.CH_2.CH_2.CO.NH.CH_2.COOH$	Non-toxic in doses of 1.5 gm. per kilo. Not so easily oxidized as phenylpropionic acid.
II	Cinnamic acid . . . . .		Less toxic than phenylpropionic acid. Readily oxidized.
	Cinnamoylglycocoll . . . . .		Non-toxic. Resistant to oxidation.

	Substance.	Result.
III }	Phenyl- $\beta$ -oxypropionic acid .....	Less toxic than phenylpropionic acid. Moderately easily oxidized.
	Phenyl- $\beta$ -oxypropionylglycocoll. ....	Non-toxic. Very resistant to oxidation.

The free acids were prepared by the usual methods. The glycocoll derivatives were synthesized by the methods recently described in this *Journal*.<sup>1</sup> The substances were administered by subcutaneous injection of the sterile solutions of the neutral sodium salts, with the exception of cinnamic acid which on account of the insolubility of the sodium salts, was neutralized with ammonia.

The results were as follows:

(1) *Phenylpropionic acid*<sup>2</sup> administered to cats in doses of 1 gram per kilo was invariably followed by death; the animal usually dying in from forty to sixty hours. Much acetophenone is found in the urine,<sup>3</sup> and it is possible that the toxicity of the acid is largely due to the formation of this intermediary product of metabolism. But little, if any, unchanged phenylpropionic acid can be detected in the urine, oxidation of the acid is therefore probably fairly complete. Half a gram per kilo given to cats or dogs is not a fatal dose and but slight poisoning symptoms are observable. The composition of the urine secreted under these conditions is qualitatively similar to that obtained when fatal doses are administered.

(2) *Phenylpropionylglycocoll* administered to cats in even larger quantities, 1.5 gram per kilo, than the corresponding

<sup>1</sup> iv, p. 431; v, p. 303.

<sup>2</sup> The doses are all recorded in terms of the free acids although they were administered in the form of their salts.

<sup>3</sup> The practical details of the analysis of the urines will be recorded in a later paper on the fate of phenylpropionic acid derivatives in the animal body.

lethal dose of phenylpropionic acid, produces no toxic symptoms whatever. The substance is excreted almost entirely unchanged and either no acetophenone or only a minute trace can be detected in the urine. When administered to dogs in dose of 0.5 gram per kilo, more than half the amount is excreted unchanged but the urine contains notable amounts of phenyl- $\beta$ -oxypropionic acid, acetophenone and hippuric acid. When given to large dogs in doses of about 0.5 gram per kilo, oxidation is more nearly complete.<sup>1</sup>

*Cinnamic acid.* Ammonium cinnamate given to cats and dogs in doses equivalent to from 0.25–0.5 gram per kilo of the free acid produced no noteworthy effects. No unchanged cinnamic acid could be detected in the urine, but a small amount of a substance which appeared to be cinnamoylglycocoll was excreted. A little phenyl- $\beta$ -oxypropionic acid and acetophenone were also detected in the urine together with much hippuric acid. Oxidation of the cinnamic acid was probably almost complete and when smaller doses than the foregoing are given the intermediary products of metabolism are completely oxidized also.

*Cinnamoylglycocoll.* The sodium salt of this acid was practically unattacked when given to cats in doses corresponding to 0.25 to 1.0 gram per kilo. About three-fourths of substance given was recovered from the urine. No signs of any toxic action were observed.

*Phenyl- $\beta$ -oxypropionic acid.* This acid was found to be more resistant to oxidation than either cinnamic acid or phenylpropionic acid. Administered to cats in doses of 0.6 gram per kilo, it was found that three-fourths could be recovered from the urine unchanged. Only minute traces of acetophenone and hippuric acid were found in the urine. When given to dogs in doses of 0.25 gram per kilo only a minute amount of unchanged acid could be detected. Acetophenone was present in fair amount and much hippuric acid. Oxidation of the oxy-acid was fairly complete.

<sup>1</sup> In a previous paper in which the fate of phenylpropionylglycocoll was recorded (this *Journal*, iv, p. 432) no evidence of an increased resistance to oxidation, as compared with phenylpropionic acid, was obtained. This was owing to the employment of insufficiently large doses.

*Phenyl- $\beta$ -oxypropionylglycocoll.* This substance was given to cats and dogs in doses of from 0.4-1.0 gram per kilo. The substance proved to be practically unattacked as almost the whole was recovered unchanged from the urine. Traces of cinnamoylglycocoll were apparently formed.



# BACILLUS INFANTILIS (n. s.) AND ITS RELATION TO INFANTILISM.

(Plates VI and VII.)

By ARTHUR I. KENDALL.

(Fellow of the Rockefeller Institute for Medical Research.)

(From the Laboratory of Dr. C. A. Herter, New York.)

(Received for publication, January 1, 1909.)

## Introduction.

The bacilli described in this paper, together with several variants were isolated from a series of chronic intestinal infections of obscure origin and etiology. The cases present a definite symptom-complex which has recently been described by Dr. C. A. Herter<sup>1</sup> and termed by him, "Infantilism from chronic intestinal infection." Clinically the pronounced symptoms are: an arrest of body development associated with only a slight retardation of cerebral development; marked abdominal distension (without, however, a noticeable enlargement of the spleen); slight to moderate secondary anæmia; marked bodily and mental fatigue, brought on by relatively slight exertion, and disturbances of intestinal function manifested usually by repeated diarrhoeal attacks and impaired powers of absorption.

Chemically, the salient features are: the presence of excessive amounts of indican and phenolic bodies in the urine; indolacetic acid occurs in some cases and may be present in greatly excessive amounts. The aromatic oxyacids may also occur in excessive quantities.

In patients receiving considerable fat in the diet, the feces contain a large excess of neutral fats, fatty acids and soaps, the excretion of the latter leading to considerable losses of calcium and magnesium to the body. This loss of calcium and magne-

<sup>1</sup> Herter: *Infantilism*, The Macmillan Company, 1908.

sium furnishes a logical explanation of the retardation of skeletal development.

*Part I: Microscopical Examination of Infantilism Stools.*

Representative fields of stools from typical cases of infantilism, stained by the Gram-Weigert method (Gram-stain followed by dilute carbol fuchsin, as a counter-stain) are strongly Gram-positive, and recall similar pictures described by Escherich<sup>1</sup> in his cases of *Blaue Bacillöse* with respect to the great diminution or even absence of Gram-negative cocco-bacilli of the coli-aërogenes type, and the dominantly Gram-positive character of the fields. Certain differences are detectable, however, upon closer scrutiny. The Gram-positive curved bacilli which are characteristic of *Blaue Bacillöse* cases are few in number or even absent in infantilism stools, while the most distinctive organisms in the latter are straight rods measuring from 0.50 to 0.75 microns in diameter, and from 2.5 to 3.75 microns in length. These bacteria are Gram-positive and occur singly or in pairs, rarely in short chains of from four to six elements. They present no distinctive peculiarities of arrangement, although they are frequently collected into rather sharply defined masses.

It is not uncommon to find signs of degeneration among these bacilli, particularly if they are derived from constipated stools.<sup>2</sup> In such instances the bacilli are more or less irregular in outline and do not stain uniformly, some portions of the cytoplasm remaining Gram-positive, while other portions stain faintly with the counter-stain, or not at all.

Cultural methods demonstrate these bacilli to be *B. infantilis*, *B. bifidus*<sup>3</sup> or *B. acidophilus*.<sup>4</sup> One type may predominate or all may be represented at the same time.

<sup>1</sup> Escherich: *Jahrb. f. Kinderheilk.*, lii, pp. 1, et seq., 1900.

<sup>2</sup> Herter and Kendall: *This Journal*, v, p. 289, 1908.

<sup>3</sup> Tissier: *La flore intestinale du nourrisson*, Paris, 1900. Tissier gave this organism the name *B. bifidus communis*, a trinomial. This name, being a trinomial, violates the law of botanical nomenclature and is incorrect. Inasmuch as the term *bifidus* expresses tersely the most prominent characteristic of the bacillus, it should be retained as the specific name, eliminating the superfluous term, *communis*.

<sup>4</sup> Moro: *Jahrb. f. Kinderheilk.*, lii, p. 38, 1900; *Wien. klin. Wochenschr.*, no. 5 1900.

It should be possible, theoretically, to make a morphological diagnosis between *B. bifidus* and *B. acidophilus* on the one hand, and *B. infantilis* on the other. Tissier claims that *B. bifidus* occurs typically with pointed ends and not infrequently one sees pairs of bacilli lying at an angle with each other—the so-called geniculate arrangement. *B. acidophilus* resembles *B. bifidus* somewhat morphologically, but the bacilli occur in sharply circumscribed masses, instead of geniculate pairs in stools. *B. infantilis*, on the contrary, has definitely rounded ends; clostridial forms are uncommon, and the bacilli do not occur in regular alignment as a rule.

The various manipulations to which stools are subjected prior to microscopical examination are usually sufficient to disturb the original orientation of the bacilli, while their morphology varies somewhat with the consistency of the stool, so that the final criteria of their differentiation and recognition must be based upon cultural methods.

Branched forms are not uncommon in the stools of infantile patients, and rarely one sees a peculiar modification of the branched form to which the term "antennate" has been applied. The branched forms may be referable to either *B. bifidus* or *B. infantilis*; each organism produces under certain undetermined conditions branched forms in stools. It is not impossible that some development has taken place after the stool was passed in such instances, because many of the bacteria show signs of degenerative changes which are known to occur when they are exposed to unfavorable conditions, outside of the body.

The "antennate" form is unusual in the feces and so far as the writer is aware, no form similar in appearance has been described for any known bacillus. The cell body is slightly spindle-shaped (clostridial) in outline and possesses a deeply staining metachromatic granule at one or both ends of the rod. The cytoplasm between these granules takes the stain feebly or even not at all, and suggests both by its position and general appearance a presporogenic body. From one, rarely both, of the metachromatic granules processes arise, which may be one, two or three in number; two is the most usual arrangement. These outgrowths are slightly curved, slender and elongated; they do not project in the same general direction as that of the long axis of the cell proper, but rather at an obtuse angle, resembling in a striking manner the antennæ of certain beetles. This resemblance is further accentuated by the irregular manner in which they take the stain, which gives them a jointed or articu-



lated appearance. The same forms have been encountered in old broth cultures of this organism, and they are probably to be regarded as involution forms. If this supposition be correct, the antennate forms have no particular significance in the life history of the bacterium.

Besides *B. infantilis*, *B. bifidus* and *B. acidophilus* one notices frequently in the feces of infantilism patients large, Gram-positive cocci which occur typically in pairs, less commonly in short chains. They measure about one micron in diameter and their ends are more or less pointed. Their elongated appearance has led the French writers to refer to them as "*Flamme de Bougie*"—an epithet which is very expressive of their peculiar morphology. These organisms are known as the "*Enterocoque*" of Thiercelin,<sup>1</sup> or the "*Micrococcus ovalis*" of Escherich,<sup>2</sup> and, according to Kruse<sup>3</sup> are identical with the *Streptococcus* of Hirsch-Libmann. Kruse proposed the name, "*Streptococcus lacticus*."

These organisms are the Gram-positive types most frequently found in the dejecta of infantilism cases, and *B. bifidus*, *B. acidophilus* and *Micrococcus ovalis* represent the acidophilic bacteria which are characteristic of well marked clinical states of this kind. It is noteworthy, as Herter<sup>4</sup> has pointed out, that the latter organisms are the dominant ones present in the stools of breast-fed infants—a fact that is of great importance in this connection because the bodily development and intestinal flora seem to be related to each other.

## Part II: Methods for Isolating *Bacillus Infantilis*.

The first culture of *Bacillus infantilis* was obtained from a stool in which they were present in unusual numbers, and no difficulty was experienced in obtaining fresh strains for nearly a month. At the end of that time, however, the bacterial flora changed and it became increasingly difficult to obtain cultures by the usual method of fishing plates.

<sup>1</sup> *Compt. rend. de la soc. de biol.*, April 15 and June 24, 1889.

<sup>2</sup> *Darmbakterien des Säuglings*, Stuttgart, p. 89, 1886.

<sup>3</sup> *Centralbl. f. Bact.*, xxxiv, p. 737, 1903.

<sup>4</sup> *Loc. cit.*

The colonies produced by *B. infantilis* are reasonably distinctive when they occur in pure culture, or when there are only a few other types of organisms in the same plate. As the number of colonies, either of the same type of organism or different organisms, increases, they lose their characteristic appearance, and it becomes increasingly difficult to make even a probable diagnosis by simple observation alone.

Again, contrary to generally accepted ideas, it has been found that not only do colonies of the same organisms vary in appearance in the same plate, but also that radically different types of bacteria produce colonies which are indistinguishable.

These differences are explainable upon two hypotheses. In the first place the rigidity of the medium has an extremely important rôle. In low percentage agar, those bacteria which are strongly motile, together with the forms that produce chains readily, will grow in colonies that are characterized by considerable lateral expansion. This is particularly the case in surface colonies. Submerged growths to a lesser extent obey the same general laws. On the other hand, particularly among the intestinal bacteria, the metabolic products of one type of organism may react by diffusion upon adjacent bacteria, resulting in a mutual inhibition of growth. The strongly acidogenic bacteria, for example, particularly when grown in media which contain fermentable sugars, will seriously interfere with the growth of alkali-producing types.

A consideration of the facts presented in the preceding pages indicates clearly the necessity of examining relatively large numbers of colonies, particularly in the study of unusual cases of intestinal disease. Obviously this is impracticable in actual work, where several cases are under observation at the same time. What is needed is a method whereby one may study the morphology of the bacteria from a representative majority of all the colonies which have developed in the various media, and to be able to return to specified colonies which through this preliminary examination have been demonstrated to consist of unusual types of bacteria.

This may be accomplished in the following manner. Plates showing from 20 to 50 well-separated colonies are marked with a wax pencil in such a way that the projections of these colonies upon the bottom of the plate are enclosed in a small ring. This procedure is kept up until a sufficient number have been selected

for examination. The colonies are then numbered in sequence, beginning with 1 and continuing until all are numbered, remembering to place a line under the figure 6 to distinguish it from the 9.

An ordinary microscopical slide is now ruled into squares, the size and number depending upon the number of colonies which it is proposed to study. A small amount of growth from each colony is emulsified in a drop of sterile water in its appropriate square, the films are dried, stained by the Gram-Weigert method and examined microscopically. This examination will indicate the general type of organism in each instance, and not infrequently demonstrate the fact that certain colonies are composed of two distinct kinds of bacteria. Having thus not only recognized the morphological peculiarities of the organisms, but also having a fair idea of the relative purity of each colony, one may select those organisms which it is desirable to study in detail, resorting to the colonies from which they were derived and subculturing them at convenience. This procedure has been very helpful and it is extremely probable that the rather large number of apparently undescribed forms which have been met with in this work, have been successfully isolated because attention has been paid to the study of large numbers of colonies which upon superficial examination show no macroscopical differences. By adopting this procedure, one may be reasonably certain that at least the dominant forms which will grow in artificial media, will be detected. In order to make a systematic investigation, however, it is absolutely necessary to use media containing either dextrose (lactose is usually better for infants' stools) or other easily fermentable carbohydrate for the fermenting and acid producing organisms, and carbohydrate-free media for the alkali producers. One may thus eliminate to a considerable extent the effects of antagonism between the acidogens and the alkali producers.

### *Part III: The Biology of Bacillus Infantilis.*

This organism is a motile, aërobic, facultative anaërobic, spore-forming, Gram-positive, non-capsulated bacillus. It produces acid in dextrose and saccharose, but no gas.

*Morphology.* The bacillus measures from 0.50 to 0.75 micron in diameter, and from 2.5 to 3.5 microns in length.

Upon solid media the bacilli appear as rather short, plump rods with rounded ends. They occur singly or in pairs, rarely in short chains. Spores are readily formed in such media, in the presence of free oxygen. In fluid media, on the other hand, spores are not formed as a rule, and in anaërobic cultures spore-formation has never been observed. This need for oxygen probably explains the inability of *B. infantilis* to form spores in the intestinal tract. Repeated attempts to obtain cultures of this organism after destruction of the vegetative cells by heating suspensions of the stool to 80° C. for 10 minutes have failed except in two instances. It should be stated that it was possible to isolate these organisms in unheated suspensions made from the same stool. The two cases in which spores were present do not necessarily invalidate this view. The stools were 24 hours old at the time they were examined and it is perfectly evident that the necessary conditions for spore formation were present.

The spores are central, oval, and cause a slight bulging of the cell; this enlargement is not great enough, however, to give the organisms the appearance of clostridia:

The bacteria found in the condensation water of agar, as well as in fluid media in general, differ conspicuously in appearance and staining<sup>1</sup> reaction from those grown upon solid media. In the latter instance the bacilli are relatively short and thick; in

<sup>1</sup> At first the difference in staining reaction between organisms derived from the slanted surface of agar, on the one hand, and the condensation water of the same culture, on the other, led to the suspicion that a contamination had occurred. Experiments definitely reproducing the phenomena were made, however, and it was discovered that this same property is common to a number of Gram-positive bacteria of intestinal origin. The differences observable are very striking in some instances. When the bacteria are kept upon artificial media for several successive transfers the variations tend to disappear and the organisms assume a uniform appearance whether they are obtained from the slanted surface or from the condensation water. It is probable that the sudden change from the intestinal environment to that of ordinary media is responsible for the lack of stability, and as the bacteria are in a weakened state, changes in their environment as slight as those existing in slanted surface and condensation water will produce noticeable responses in bacterial growth.

the former they are longer, thinner, and conspicuously Gram-negative. The staining reaction has been described previously in this paper, and it will suffice to say that the "punctate" appearance which one sees in stools can be reproduced exactly in fluid media.<sup>1</sup>

*Temperature relations.* *B. infantilis* grows most characteristically and luxuriantly at the body temperature; at lower temperatures the growth is scanty, and at 18° C. it practically ceases.

*Anaërobiosis.* The organism is an aërobe, although it can develop in hydrogen, carbon dioxide and in the depth of anaërobic stab cultures. Certain peculiarities concerning its oxygen needs will be described later on, in the section upon fermentation.

*Agar slant.* Upon dextrose agar (slanted) there is produced a spreading, gray, smooth, opaque, shining layer. In the condensation water growth is abundant and usually associated with the production of a pellicle. The medium becomes brown in older cultures and exhibits a tendency to the production of a well marked opacity. These two phenomena are distinct and apparently have no relation to each other.

Three variants have been noticed. The first is characterized by the production of a viscosity due to the ability of the bacteria to form adhesive, easily drawn out threads. The second variant grows poorly upon the slanted surface, and appears as a delicate, translucent, shining filiform layer which never produces the browning of the medium referred to above. Organisms of this type are chemically and morphologically identical with the luxuriantly growing varieties. They may be regarded as strains which have not become thoroughly habituated to artificial media. It should be stated that the latter type grows better in hydrogen than in aërobic conditions. The third variant is a chromogen. The pigment, which is soluble in the medium, and occurs in the upper layers, where oxygen is present, is red-brown, recalling the pigment produced by an organism erroneously named *B. lactis erythrogenes*.

The chromogenic variant may arise spontaneously. In fact

<sup>1</sup> Prolonged cultivation in artificial media causes the organism to lose its ability to take the Gram-stain; in this respect it agrees with several well known bacteria of similar origin.

a chromogenic strain has never been isolated from infantilism stools. Culturally, morphologically and chemically these strains are identical with typical infantilis cultures.<sup>1</sup>

*Gelatin stab.* Growth in gelatin stabs is scanty. In dextrose gelatin it is more vigorous. At temperatures ranging from 18° to 22° C. development takes place very slowly, and there is no appreciable liquefaction. As the temperature rises, however, the organisms develop more rapidly, and at 24° liquefaction may take place at the end of six days. The liquefaction is very slow and frequently is manifested merely by the presence of a slight infundibuliform, dry depression. Evaporation usually progresses parallel to peptonization, so that no fluid remains in the cavity. Rarely a small amount of liquid remains. In cultures which have been kept at room temperature for several months liquefaction may progress to such an extent that a funnel-shaped depression measuring half a centimeter in depth may result. The medium is not favorable for the development of the bacillus, however, and certain strains do not liquefy at all, but produce merely a softening of the medium. The reaction becomes alkaline.

*Milk.* Freshly isolated strains produce a transient acidity followed by a return to the neutral point. In older cultures the reaction becomes alkaline. The primary acidity is doubtless due to the fermentation of carbohydrates (hexoses) present in milk, due to the hydrolysis of lactose. Prolonged cultivation of the bacteria in milk is associated with more marked changes in the medium. A gelatinous coagulum is formed which is not marked and which usually requires a boiling temperature to demonstrate. This stage is followed by a gradual solution of the coagulum, an increased alkalinity and a gradual thinning of the whole medium. The thin, alkaline fluid resembles that produced by some varieties of the paratyphoid bacillus.

<sup>1</sup> Beijerinck (*Kon. Akad. von Wetenschappen te Amsterdam*, Oct. 27, 1900, and Rodet (*De la variabilité dans les microbes*, Paris, 1894) have studied the question of bacterial variation, and define three types, Degeneration, Transformation and Variation, in the narrow sense. The production *de novo* of pigment in *Bacillus infantilis* seems to correspond with the latter type of variation, which is defined as "the assumption of a new characteristic (by a single individual of a single strain) which remains constant. Insufficient or improper media, or the excess of excretory products in old cultures may be the cause of this phenomenon."

Milk is not, however, a favorable medium in which to cultivate *Bacillus infantilis* and the changes produced by this organism in milk are not distinctive.

*Potato.* The growth is luxuriant, raised, shining, smooth, brownish and is attended with a darkening of the potato. The reaction is not appreciably changed.

*Fermentation media.* Dextrose and saccharose are fermented with the production of acid, but no gas. Lactose is only slightly attacked. The acidity reaches its maximum about the fourth day, and after that time there is a gradual return to the neutral point, or even in extreme cases to alkalinity. The explanation is probably to be correlated with the ability of this organism to produce ammonia and primary amines. During the first few days there is a marked growth in the closed arm (in dextrose and saccharose fermentation tubes) and it is during this period that the acidity rapidly increases. At the end of the time specified the bacteria grow more abundantly in the open arm of the tube and this aërobic growth is associated with a large ammonia production.

Lactose fermentation media are not favorable to anaërobic growth of *B. infantilis* and it is a noteworthy fact that in the closed arm growth is practically wanting. There is, however, evidence that the organisms are proliferating.<sup>1</sup> A pellicle makes its appearance within 30 hours after inoculation, which suggests that the organism cannot derive its oxygen through the combustion of lactose, although it can thrive in an atmosphere devoid of oxygen if either dextrose or saccharose is present. Confirmation of this view is indicated by the formation of a similar pellicle in plain broth. Here again there is no substance from which the organism can derive its oxygen. Hence it is forced to obtain it from the air. The wrinkling of the pellicle, and the almost total absence of turbidity (the organism is actively motile) point strongly to the correctness of this explanation.

*Plate cultures.* Although *B. infantilis* is an aërobic organism,

<sup>1</sup> This inability to develop in lactose is significant when one considers that this sugar rather than dextrose or saccharose is the important, carbohydrate in the dietary of young children. The normal intestinal bacteria on the other hand are able to grow luxuriantly in media containing lactose.

it usually grows beneath the surface in dextrose agar plates. (Plain agar plates, on the contrary, show relatively more surface colonies.) The submerged colonies are lenticular, oval or even round. They are opaque, yellowish or gray (depending upon the depth below the surface) with delicately ciliate edges. The colonies are surrounded by an opaque area which resembles a halo. Microscopically the edges are seen to be filamentous and the general look of the colony suggests an aggregation of filaments, presenting a floccose appearance. Klatsch preparations, however, do not show the presence of long filaments; the individual bacteria, on the contrary, have a disjointed appearance and even short chains are uncommon. Surface colonies are translucent to opaque, and round, with irregular edges. Their appearance depends largely upon the relative density of the medium and particularly upon the amount of moisture present. If the latter is excessive, the colonies spread and resemble delicate films. The colonies are not characteristic, particularly when there are several different kinds of bacteria present in the same plate, and it is only by examining individual colonies microscopically that one can safely make the diagnosis.

*Biochemistry.* The biochemistry will be discussed in detail in a subsequent paper. A summary of the principal products, however, is here appended for completeness.

*Bacillus infantilis* is a powerful alkali-producer. Ammonia and an unidentified primary amine are the chief basic bodies found in cultures. The organism does not produce indol, skatol, phenolic bodies, mercaptan, hydrogen sulphide, alcohol, acetone or aldehyde. It produces lactic and succinic acids from dextrose, together with small amounts of volatile acids of unknown compositions. It gives the Voges-Proskauer reaction<sup>1</sup> in peptone media.

Certain strains of *Bacillus infantilis* are, at the time of their isolation, strongly acidophilic, or, better, have acquired the ability to withstand unusual amounts of acid. A sharp distinction should be drawn between bacteria which grow well in moderate amounts of acid, e. g., *B. acidophilus*, and those organisms which—originally not acidophilic—become by changes

<sup>1</sup> Harden and Walpole (*Proc. Roy. Soc.*, lxxxvii, p. 424, 1906) have shown that this reaction is due to the presence of methylacetylcarbinol.



in their environment able to withstand and to develop in great amounts of acid. Several cultures were obtained from a case of infantilism in association with *Bacillus acidophilus* and *Micrococcus ovalis*. All of these organisms were able to develop in a broth medium containing acetic acid of such a strength that three cubic centimeters of normal sodium hydroxide were required to neutralize one hundred cubic centimeters of the medium. The organisms were obtained in the following manner. A small amount of feces was emulsified in a tube of the acetic acid broth, and incubated two days. A loopful of the first broth culture was introduced into a second tube; after two days a third culture was prepared from the second. At the end of the last period of two days, the organisms were plated out and cultures were obtained in the usual manner. There can be no doubt that there was a decided development of *B. infantilis* during the progress of the experiment and considerable numbers of colonies were obtained on the plates. Subsequent cultural and chemical studies showed that they were identical with the first strains of this organism which had been isolated in the usual manner, in neutral media. The striking fact is that two transfers in ordinary media so changed the acid-resisting ability of the "acid infantilis" that it would not develop in media having an acidity greater than that corresponding to 1.25 cc. normal acid per 100 cc.<sup>1</sup>

<sup>1</sup> This experiment has a distinct value in relation to the question of bacterial conditions obtaining in the intestinal tract. In no other pathological conditions are the bacterial flora so complex and dependent in their ensemble upon obscure changes in environment. These environmental changes may consist of altered intestinal secretions, changes in diet or of bacterial antagonism and symbiosis or of combinations of these factors. Apparently slight nutritional alterations are frequently accompanied by surprising modifications in the bacterial behavior, the bacterial response seeming to be out of proportion to the intensity of the stimuli.

Although we are ignorant of the fundamental principles which dominate the bacterial flora of the intestinal tract, evidence is slowly accumulating which points to a symbiotic relationship between the host on the one hand, and the dominant types of bacteria on the other, at least in normal, healthy individuals. Undoubtedly diet plays a prominent part in determining which bacteria shall increase and which shall be inhibited.

When one stops to consider the extraordinary number of bacteria, dead and living, that are voided every day in the feces, and compares

The discovery of the acidophoric strain of *B. infantilis* brought up the question of antagonistic and symbiotic relations between this organism and bacteria with which it is commonly associated in the intestinal tract. Naturally it is impossible to reproduce conditions comparable to those existing in the intestines, but attempts have been made to compare the behavior of pure cultures of a few representative bacteria with combinations of the same bacteria grown together.

*Bacillus coli* and *Micrococcus ovalis* (enterococcus) have been chosen as the types to compare with *B. infantilis*, and all observations have been made in fermentation tubes, where every transition can be obtained from almost absolute anaërobiosis to complete saturation with free oxygen.

this figure with the relatively few organisms which are ingested during the same period, some idea of the enormous proliferation which takes place in the intestinal tract will be obtained.

Escherich, Tissier and Moro have studied the kinds of bacteria present in the dejecta of normal nurslings, and find that certain well defined types of organisms are regularly present to the more or less complete exclusion of other types. Tissier has noticed that the vast majority of these bacteria are acidophilic, and he believes that the high degree of acidity which they can resist while still thriving acts as a deterrent to the growth of foreign organisms. When an infant suffers from an intestinal upset, the normal bacterial conditions are disturbed, and new varieties make their appearance. As conditions return to the normal, there is a gradual corresponding reappearance of the customary flora, associated with the disappearance of the abnormal types.

If, however, the abnormal conditions persist, the bacteria associated with the change may become habituated in the intestine, and eventually may replace, in part at least, the normal inhabitants. This invasion, as the writer has noticed in a number of instances, is accompanied by reciprocal modifications in both the normal and the invading organisms. This change is manifested by the ability of the two types of organisms to grow in the presence of each other, even if originally this was impossible. One may demonstrate this fact by comparing corresponding strains from normal intestines with the "modified" varieties. Usually one finds that the "normal" organisms will not thrive in such cultures.

This fact points to deep seated modifications in the biology of both the invading bacteria and the normal obligate organisms and undoubtedly these mutual adaptations explain in part the persistence of unusual types of bacteria in chronic intestinal diseases.

The facts brought forward indicate the necessity of studying with great thoroughness those cases of intestinal derangement accompanied by evidences of abnormal bacterial development.

Protocols of two experiments are reproduced. The experiments were carried on during four days, both in dextrose and actose. All of the bacteria used in these experiments were given preliminary cultivation in dextrose broth to insure a high degree of reproductive growth.

These experiments emphasize once again the inability of *B. infantilis* to ferment lactose. This is indicated not only by the absence of turbidity in the closed arm of the fermentation tube, but by the production of a thick pellicle upon the free surface of the medium in the bulb. There is a corresponding absence of inhibitory action of this organism upon *B. coli* and *Micrococcus ovalis*, although in dextrose under similar conditions the restraining activity is marked.

It is obvious, then, that the marked decrease in gas formation which is observed in cases of infantilism is due to some factor other than the mere presence of *B. infantilis* because this reduction is as marked in lactose as in dextrose. It is extremely probable that *B. bifidus* is instrumental in preventing the development of bacilli of the colon-aërogenes types, and inasmuch as the latter organisms are the dominant gas-formers of the intestinal tracts of young children, it is logical to associate the comparative absence of the gas-formers and the simultaneous presence of *B. bifidus* with the non-appearance of gas by the fecal bacteria from well marked infantilism cases.

Unfortunately the rôle of *B. bifidus* in this connection must, for the present at least, rest upon purely circumstantial evidence. It is not possible with our present methods to prepare media in which *B. bifidus* and *B. coli* (or other facultative organisms) shall under the same conditions grow with the same relative intensity. Obviously, if one organism develops more rapidly than the others it is out of the question to draw correct conclusions with reference to their antagonistic properties.

*The systematic position of B. infantilis.* *B. infantilis* differs in essential characteristics from any previously described organism known to the writer. Particular attention has been paid to the previously published descriptions of bacteria found in nurslings' stools, both in health and disease, and the works of Tissier,<sup>1</sup>

<sup>1</sup> *Loc cit.*, p. 263, 1900.

## EXPERIMENT 1.

Culture.	Day.	DEXTROSE.				LACTOSE.			
		Gas in mm.	Turb. elsd. arm.	React.	Pell.	Gas in mm.	Turb. elsd. arm.	React.	Pell.
Infantilis.....	1	-	+	acid	-	-	-	±	++
	2	-	+	"	-	-	-	±	++
	3	-	+	"	+	-	-	alk.	++
	4	-	+	+1.2	+	-	-	- 0.5	++
Coli.....	1	16	+	acid	-	15	+	acid	-
	2	18	+	"	-	20	+	"	-
	3	22	+	"	-	24	+	"	-
	4	22	+	+2.25	-	25	+	+1.5	-
Ovalis.....	1	-	+	acid	-	-	+	acid	-
	2	-	+	"	-	-	+	"	-
	3	-	+	"	-	-	+	"	-
	4	-	+	+1.15	-	-	+	+1.1	-
Infantilis and Coli..	1	12*	+	acid	-	11	+	acid	-
	2	12	+	"	-	27	+	"	-
	3	12	+	"	-	32	+	"	-
	4	12	+	+2.2	-	32	+	+1.6	-
Infantilis and Ovalis	1	-	+	acid	-	-	+	acid	-
	2	-	+	"	-	-	+	"	-
	3	-	+	"	-	-	+	"	-
	4	-	+	+2.5	-	-	+	+3.0	-
Coli and Ovalis....	1	22	+	acid	-	16	+	acid	-
	2	23	+	"	-	26	+	"	-
	3	24	+	"	-	27	+	"	-
	4	20	+	+3.4	-	25	+	+3.1	-
Infantilis, coli and Ovalis.....	1	12	+	acid	-	19	+	acid	-
	2	11	+	"	-	21	+	"	-
	3	11	+	"	-	21	+	"	-
	4	10	+	+3.5	-	21	+	+3.3	-

\* Trials with a freshly isolated culture of *B. infantilis* showed a much greater inhibition of gas production. The total volume of gas produced in the preliminary experiments in those tubes containing *B. coli* and *B. infantilis* only showed 5 mm. of gas, although *B. coli* alone produced 26 mm. in the same media.

## EXPERIMENT II.

Culture.	Day.	DEXTROSE.				LACTOSE.			
		Gas in mm.	Turb. elsd. arm.	React.	Pell.	Gas in mm.	Turb. elsd. arm.	React.	Pell.
Infantilis .....	1	—	+	acid	—	—	—	±	++
	2	—	+	"	—	—	—	alk	++
	3	—	+	"	+	—	—	"	++
	4	—	+	+1.3	+	—	±	-0.6	++
Coli.....	1	26	+	acid	—	35	+	acid	—
	2	29	+	"	—	37	+	"	—
	3	28	+	"	—	36	+	"	—
	4	28	+	+2.45	—	34	+	+3.0	—
Ovalis.....	1	—	+	acid	—	—	+	acid	—
	2	—	+	"	—	—	+	"	—
	3	—	+	"	—	—	+	"	—
	4	—	+	+1.05	—	—	+	+1.50	—
Infantilis and Coli..	1	21	+	acid	—	29	+	acid	—
	2	23	+	"	—	33	+	"	—
	3	22	+	"	—	31	+	"	—
	4	22	+	+2.20	—	31	+	+2.65	—
Infantilis and Ovalis	1	—	+	acid	—	—	+	acid	—
	2	—	+	"	—	—	+	"	—
	3	—	+	"	—	—	+	"	—
	4	—	+	+2.7	—	—	+	+2.8	—
Coli and Ovalis .....	1	25	+	acid	—	28	+	acid	—
	2	26	+	"	—	32	+	"	—
	3	22	+	"	—	30	+	"	—
	4	22	+	+3.8	—	30	+	+3.0	—
Infantilis, Coli Ovalis.....	1	11	+	acid	—	30	+	acid	—
	2	11	+	"	—	31	+	"	—
	3	9	+	"	—	28	+	"	—
	4	10	+	+3.45	—	28	+	+3.9	—

Escherich,<sup>1</sup> Finkelstein,<sup>2</sup> Moro<sup>3</sup> and Salge,<sup>4</sup> have been freely consulted.

An organism described by Salge attracted especial attention. He found it in cases of catarrh of the small intestine and discovered that it had the property of breaking up sodium oleate into lower fatty acids, and that the presence of fats increased its fermentative powers. Inasmuch as an abnormal excretion of fatty acids and fats is a feature of infantilism, a possible relationship between infantilis and Salge's bacillus was suspected. Subsequent investigation, however, showed that there is very little resemblance between the two organisms.

Finkelstein described an acidophilic organism which he obtained from cases of *Blaue Bacilliose*, but it is evident that his bacillus is closely related to *B. acidophilus*. (This organism, as already pointed out, occurs in infantilism stools together with *B. infantilis*.)

*B. infantilis* belongs to the *B. subtilis* group. It produces resistant spores, forms alkali in milk and non-saccharine media, and does not produce gas. It is, however, smaller than any hitherto described subtiloid bacillus, and its ability to liquefy gelatin is much less marked than is the case with other members of this group. It should be mentioned in this connection that an organism having the morphological and cultural characters of *B. infantilis* has been isolated by the writer from canned plums. It differs from *B. infantilis*, however, by its relatively rapid peptonizing action in gelatin and its inability to form primary amines. This organism seems to be a connecting link which not only indicates the relation of *B. infantilis* to *B. subtilis* group but emphasizes the lines along which the latter organism tends to deviate from the typical organisms of the group.

#### Part IV. The Relation of *B. Infantilis* to Infantilism.

*B. infantilis* is a spore-forming organism, and as Theobald Smith<sup>5</sup> has pointed out, spore-forming bacteria are not, as a rule,

<sup>1</sup> *Loc. cit.*

<sup>2</sup> *Deutsch. med. Wochenschr.*, p. 263, 1900.

<sup>3</sup> *Loc. cit.*

<sup>4</sup> *Jahrb. f. Kinderheilk.*, lix, p. 399, 1904.

<sup>5</sup> Theobald Smith: Some Problems in the Life History of Pathogenic Microorganisms, *Amer. Med.*, viii, pp. 711-718, 1904.

obligate parasites, at least in man. The organism has not only been found in the stools of all the typical cases of infantilism so far examined<sup>1</sup> but also in the dejecta of normal nurslings derived from various sources, although in the latter instances the bacilli were very few in number and were not obtained from all the feces examined.

The fact that these cases, both pathological and normal, represent a fairly wide geographical area seems to indicate that *B. infantilis* may be well distributed in nature. In some instances it apparently finds a favorable environment in the intestinal tract of young children, obtains a foothold and proliferates there. In spite of this proliferation, however, specific agglutinins are apparently not produced, and although it certainly occurs in large numbers at certain periods of the disease, evidence is strongly in favor of the view that it is non-invasive.

One is not justified, however, upon these grounds in concluding that there is no etiological relationship. Feeding experiments upon a dog resulted in the establishment of a well-marked diarrhoea<sup>2</sup> associated with the appearance of *B. infantilis* in the stools. A monkey, whose diet was carefully regulated, reacted even more strikingly. The movements became soft, pale in color, strongly Gram-positive, and there was simultaneously a marked increase of acidogenic bacteria (first of the *Micrococcus ovalis* type, then a rapid rise in the proportion of *B. acidophilus*, associated with a moderate number of *B. bifidus*). There was a corresponding diminution in the Gram-negative coli-aërogenes type of bacilli. That is, there was bacterially a decided tendency toward the development of the infantilism type of stool.<sup>3</sup> These effects are

<sup>1</sup> For list of cases, see Herter, *loc. cit.*

<sup>2</sup> It is interesting to note in this connection that Ardoïn (Thèse de Paris, p. 78, 1898) and Spielgelberg (*Jahrb. f. Kinderheilk.*, xlix, p. 194, 1895) have isolated and described bacteria belonging to the subtilis group which cause decided diarrhoeal disturbances, particularly in young children. Certain cases quoted by these investigators were characterized by the relative abundance of subtiloid organisms in the stools.

<sup>3</sup> The appearance of acidophilic bacteria following so closely upon that of *B. infantilis* is perhaps the most noteworthy feature of these feeding experiments, because cases of infantilism usually run the same course, bacterially. The explanation of this bacterial sequence is not known and the data available at the present time do not justify more than the bare statement of the fact.

transient and tend to disappear after a few days, but they may be reproduced by fresh infection with *B. infantilis*. These diarrhoeal disturbances are conceivably due to the irritant action of the products produced *in situ* by *B. infantilis*, and the experiences of Ardoin<sup>1</sup> and Spiegelberg<sup>2</sup> certainly are in favor of this view.

#### SUMMARY.

(1) A spore-forming organism, *B. infantilis*, described above, has been isolated from each of a series of cases of infantilism.

(2) It has also been found in limited numbers in the feces of some although not all normal infants.

(3) *B. infantilis* is not an obligate intestinal bacillus, but a saprophytic organism which under certain undetermined conditions finds a suitable environment in the intestinal tract and proliferates there.

(4) It produces no agglutinins and there is no direct evidence indicative of its etiological relationship to infantilism.

(5) *B. infantilis* fed to a dog and a monkey caused in each animal a pronounced softening of the stools and diarrhoea. In the monkey, this diarrhoea was followed by a decided diminution in the Gram-negative gas-producing bacilli of the coli-aërogenes type, and a noteworthy increase in the Gram-positive acidophilic flora. *B. bifidus* in moderate numbers and *B. acidophilus* in excessive numbers were the dominant organisms. There was a gradual return to the normal type of stool, both macroscopically and microscopically.

(6) These experiments furnish evidence in favor of the view that the diarrhoea observed in cases of infantilism may be caused by irritant metabolic products resulting from the proliferation of *B. infantilis* in the intestinal tract.

In conclusion the writer wishes to express his indebtedness to Dr. C. A. Herter not only for the opportunity of studying these cases but also for many helpful suggestions and advice during the progress of the work. The photographs which accompany this paper were made by Dr. Leaming of the Rockefeller Institute, to whom the writer is indebted.

<sup>1</sup> *Loc. cit.*

<sup>2</sup> *Loc. cit.*



## EXPLANATION OF THE PLATES.

1. Pure culture of *B. infantilis*, showing Gram-positive and "punctate" forms, the latter staining faintly. *a* shows an antennate form ( $\times 1000$ ).
2. Submerged colonies of *B. infantilis*, showing the floccose structure.
3. A typical field from an infantilism stool. The principal organisms represented are: *B. infantilis*, *B. acidophilus*, *B. bifidus*, *Mic. ovalis*, *B. coli*. The doubly contoured bodies, resembling large capsulated cocci are relatively common in typical infantilism stools. Their significance is unknown ( $\times 1000$ ).
4. A typical dextrose fermentation-tube sediment from a case of infantilism. I-IV, VI, *B. bifidus*; II, pseudo-branched form of *Mic. ovalis*; III, club-shaped form of *B. bifidus*; V, antennate form of *B. infantilis* ( $\times 1000$ ).

The diagnosis of these organisms is based upon the cultural study of the stool from which the photograph was prepared.



FIG. 1



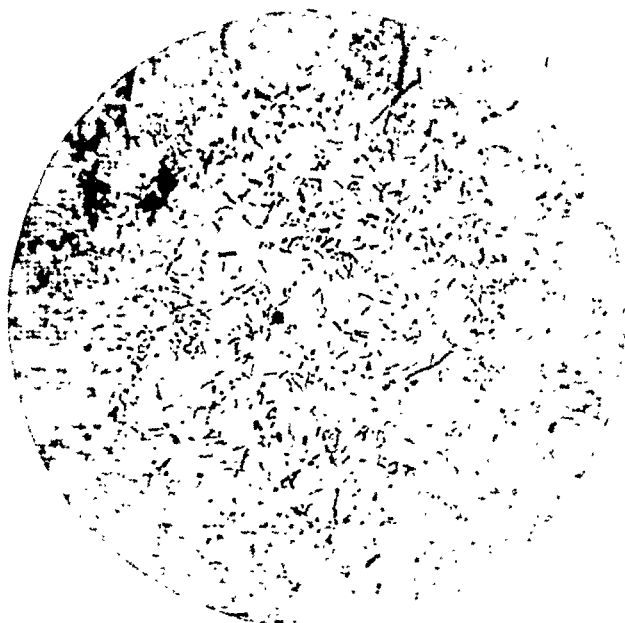
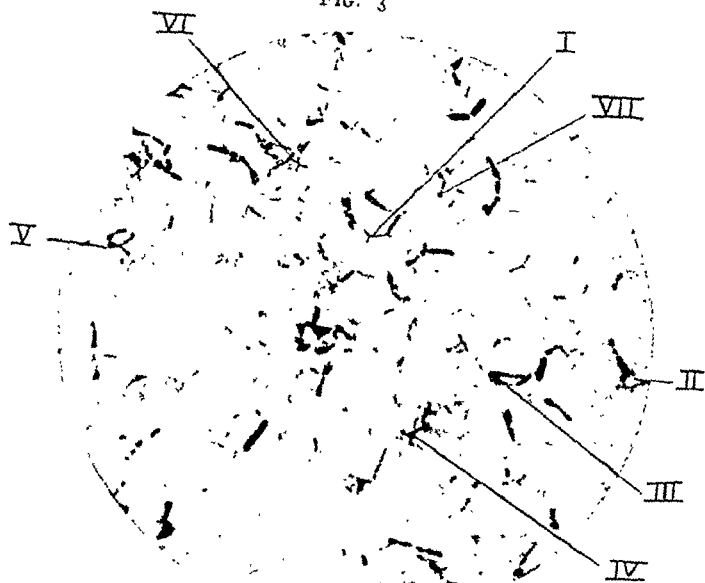


FIG. 3





# NOTE ON THE PRODUCTS OF BACILLUS INFANTILIS GROWN IN ARTIFICIAL MEDIA.

BY

C. A. HERTER

AND

A. I. KENDALL

(Fellow of the Rockefeller Institute.)

(Received for publication, December 12, 1908.)

Bacteriological studies of the feces in certain cases of arrested development in infancy<sup>1</sup> have shown this disorder to be associated with the presence of large numbers of the microorganisms to which we have given the name *B. infantilis*. Although the relation of this microorganism to the derangement of intestinal function is not yet clear it is evident that it is so prominent among the intestinal bacteria in this disease, even in its early stage, as to deserve careful study from every standpoint. The morphological and cultural characters of *B. infantilis* have been studied by one of us (Kendall) and we now desire to record here some observations which have been made on the bio-chemical properties of the bacillus.

The most noteworthy fact relating to the biological chemistry of *B. infantilis* is its ability to form volatile alkali. When grown for two weeks in plain broth at the body temperature this organism was found to have given rise to considerable quantities of volatile bases, consisting chiefly of ammonia. The quantity of volatile base formed in three different experiments carried on under approximately the same conditions was equivalent to 3.8 per cent, 4.2 per cent and 4.1 per cent normal sodium hydroxide, using alizarin as an indicator. It is interesting to observe that the formation of volatile alkali by *B. infantilis* was from three to four times greater than the amount made in plain broth by *B. coli* growing under essentially the same conditions for the same length of time. While the greater part

<sup>1</sup> See *Infantilism from Chronic Intestinal Infection*, by C. A. Herter, The Macmillan Company, 1908.

of the volatile bases formed by *B. infantilis* is neutralized by acids simultaneously formed (lactic and succinic, together with volatile acids, probably for the most part propionic and butyric) the bases after a few days predominate sufficiently to impart a decided alkaline reaction to the broth culture.

We are certainly justified in classing *B. infantilis* as one of the very active producers of ammonia. The differences just mentioned respecting the ability of *B. coli* and *B. infantilis* to make volatile bases are not attributable to differences in the growth of the bacteria in the two sets of culture. For while it is not possible to gauge with accuracy the inequalities in rapidity of growth, a comparison of the turbidities, as well as of the microscopical appearances, indicated that we were dealing with fairly comparable rates of reproduction, as shown by the conditions at the end of the incubation period of two weeks.

The volatile alkali obtainable from broth cultures of *B. infantilis* does not consist entirely of ammonia. The use of Hoffmann's carbylamine reaction showed clearly that a primary amine is formed early in the course of the decomposition. The development of an unmistakable carbylamine reaction has been a feature of all our alkaline distillates obtained from broth cultures of *B. infantilis*. We are disposed to attribute this reaction to the presence of an alkylamine but it cannot be denied that the presence of diamines such as putrescine and cadaverine is not impossible in our distillates. Methylamine and ethylamine probably do not occur separately among decomposition mixtures and it is not unlikely that both are present in our distillates, the former perhaps preponderating. We have made no observations with a view to determining the amount of primary amines formed by *B. infantilis*.

In order to determine whether *B. infantilis* causes the putrefactive decomposition of proteids it was grown in broth for a period of three weeks or longer. The cultivation was carried on in flasks containing one liter of the culture medium and under such conditions as were likely to secure both aerobic and anaerobic development. No attempt was made, however, to secure such strict anaerobic conditions as are obtainable under hydrogen or under carbon dioxide. As there is a strong tendency for the organisms to collect on the surface of the culture medium

there may be some difficulty in securing good growth in the lowest part of the flask where the conditions are relatively anaerobic, but this difficulty was in a degree overcome by frequently shaking the receptacles. Under these experimental conditions we were unable to detect the presence, of indol, skatol, phenol, aromatic oxyacids, hydrogen sulphide or mercaptans. From acidified concentrated broth cultures and from milk cultures it was possible to obtain ethereal extracts containing material which gave the color reactions for indolacetic acid, but this derivative of tryptophan was not thus obtainable in amounts sufficient for identification. Assuming that we are justified from the color reactions in considering that indolacetic acid was formed, it is certain that it was present in only very slight concentration.

The addition of tryptophan to the broth medium did not yield indol or skatol from the action of *B. infantilis* nor did this addition lead to the formation of an increased amount of indolacetic acid. Similarly, the addition of tyrosin to the broth did not lead to the development of phenolic derivatives of tyrosin. Finally, the addition of cystin to the broth was not followed by the liberation of hydrogen sulphide or methyl mercaptan. From these experiments we have reached the conclusion that our organism does not possess putrefactive activities, at least under ordinary conditions of growth. But it is proper to say that different results may conceivably be obtained under strict anaerobic conditions. It is also possible that under states of symbiotic action with other bacteria *B. infantilis* may develop powers different from those which we have described. It is apparently a characteristic of infantilism from intestinal infection that the urine gives very strong reactions for aromatic oxyacids and we do not consider it impossible that our microorganism has a part in their formation, although the indications are at present opposed to this view.

When boiled with caustic potash or caustic soda the broth cultures of *B. infantilis* yield a strong reddish-brown color which corresponds to the characters of the Voges-Proskauer reaction. This reaction is only obtainable from cultures grown on media containing peptones or albumoses.

On media containing dextrose *B. infantilis* forms lactic acid,



succinic acid and volatile fatty acids. We have not detected the presence of alcohols, ketones or aldehydes.

It is a question of some interest whether so strong an alkali producer as *B. infantilis* may, by virtue of its production of ammonia, give rise to significant irritant action on the intestinal mucous membrane in those cases where the organism is in process of becoming parasitic and is present in very large numbers.

Finally it should be observed that the ether extract of old cultures of *B. infantilis* in broth, yields an abundance of an apparently fatty substance which we deem worthy of further study.

# A COMPARISON OF THE METHODS OF REID AND SCHENCK FOR QUANTITATIVE ESTIMATION OF THE REDUCING SUBSTANCES IN BLOOD.

By J. J. R. MACLEOD.

(From the Physiological Laboratory, Western Reserve University,  
Cleveland, O.)

(Received for publication, November 20, 1908.)

A critical examination of the various methods which, up to that time, had been suggested for the estimation of the reducing power of blood was made by Seegen<sup>1</sup> in 1892. In 1894, Schenck,<sup>2</sup> partly in reply to Seegen's criticisms of a method suggested by him, published a detailed account of this method and supplied evidence of its accuracy. Two years later, Waymouth Reid<sup>3</sup> published another method which he showed to be of great accuracy. The prime difficulty in making an estimation of the reducing substances in blood resides in the removal of the proteid, and it is in the exact process employed for this that the methods essentially differ. Since 1896 various other ways have been suggested for removal of the proteid, among which may be mentioned that of colloidal precipitation by means of dialyzed iron (Michaelis and Rona<sup>4</sup> and Oppler and Rona<sup>5</sup>) and that of dialysis of the blood against sterile isotonic saline at ice cold temperature (Edie and Spence<sup>6</sup>).

In connection with researches carried on during the past two years in this laboratory, the method of Waymouth Reid has been that employed, but more recently parallel determinations have been made by the method of Schenck. The object of making this comparison was to see whether Schenck's method is as reliable as that of Reid for, if so, considerable expense and time

<sup>1</sup> J. Seegen: *Zentralbl. f. Physiol.*, vi, p. 501, 1892; vii, p. 604, 1893.

<sup>2</sup> Schenck: *Arch. f. d. ges. Physiol.*, iv, p. 191, 1894.

<sup>3</sup> Waymouth Reid: *Journ. of Physiol.*, xx, p. 316, 1896.

<sup>4</sup> L. Michaelis and P. Rona: *Biochem. Zeitschr.*, viii, p. 356, 1908.

<sup>5</sup> Oppler and Rona: *Ibid.*, xiii, p. 121, 1908.

<sup>6</sup> Edie and Spence: *Biochem. Journ.*, ii, p. 103, 1907.

could be saved. Another reason for making the comparison lay in the fact that in a recent research by Liefmann and Stern,<sup>1</sup> in which the reducing power of the normal blood of man was determined by Schenck's method, much lower values were obtained than by the older investigators who used one or other of the older methods. Thus, the average percentage reducing power for human blood found by Liefmann and Stern is 0.08, that given by Seegen 0.17, by Frerichs 0.12 to 0.33, by von Mering 0.1 to 0.15, by von Noorden 0.05 to 0.15 and by Naunyn 0.08 to 0.09.

In dog's blood, removed immediately after etherizing, Vosburgh and Richards<sup>2</sup> found by the use of Waymouth Reid's method, the normal percentage of reducing substance to be 0.17 (16 results). (In this average, unusually high figures are omitted.) Underhill<sup>3</sup> by the same method, found an average of 0.16 (16 results), which is about the same as that obtained by me.<sup>4</sup> By the use of Schenck's method, Embden, Lüthje and Liefmann<sup>5</sup> found the normal percentage of reducing substance in dog's blood removed without anæsthesia from the jugular vein to vary considerably with the temperature of the room in which the dog had been kept. The average percentage at a temperature between 5° and 10° C. was about 0.098, and for higher temperatures it was less. It must be remembered, firstly, that this is for venous blood which, as is well known, contains less reducing substance than does arterial, and, second, that no ether was used. On account of the discrepancies it was thought advisable to make a comparison of the amounts of reducing substance found in the same specimen of blood by the two methods.

A brief description of these two newer methods, as employed by me, may not be out of place.

*Waymouth Reid's method.* The blood is delivered directly from the artery into a weighed beaker containing 250 cc. of a solution of 7 per cent

<sup>1</sup> Liefmann and Stern: *Biochem. Zeitschr.*, i, p. 291, 1906.

<sup>2</sup> Vosburgh and Richards: *Amer. Journ. of Physiol.*, ix, p. 38, 1903.

<sup>3</sup> F. P. Underhill: *This Journal*, i, p. 113, 1905.

<sup>4</sup> J. J. R. Macleod: *Amer. Journ. of Physiol.*, xix, p. 388, 1907; xxii, p. 373, 1908.

<sup>5</sup> Embden, Lüthje and Liefmann: *Beitr. z. chem. Physiol. u. Pathol.*, x, p. 265, 1907.

phosphotungstic acid and 2 per cent hydrochloric acid. After weighing, to ascertain the amount of blood taken, the beaker is placed on a sand bath and its contents heated to boiling, and the boiling continued (two or three minutes) until the proteid precipitate collects into a hard crumbly mass. After cooling, the clear supernatant fluid is filtered off, nearly neutralized to litmus (left just acid) and placed in an evaporating dish on a water bath. The precipitate is transferred to a mortar and ground into a chocolate-like paste to which is then added, with constant rubbing, a large amount of cold water, the resulting suspension being then thrown on to a suction filter and sucked dry. The washings are nearly neutralized to litmus (but left just acid) and added to the evaporating dish containing the original filtrate. The washed precipitate is transferred (along with the filter paper) to the mortar and again rubbed up with a large amount of water and filtered under suction, the filtrate after partial neutralization being added to the contents of the evaporating dish. The same process is repeated a third time. The contents of the evaporating dish are then evaporated to a bulk of about 40 cc. exactly neutralized towards litmus paper and filtered through ash-free thick filter paper into a 100 cc. graduate, the evaporating dish being washed on to the filter. The total volume of the filtrate and washings is made up to 85 cc. which is then mixed in a beaker with 30 cc. each of the two constituent solutions of Allihn's reagent. The beaker, covered with an inverted Petrie's capsule, is passed through a retort ring and suspended by its rim into a briskly boiling water bath for exactly half an hour, after which the beaker is removed from the water bath and 130 cc. of water are added to its contents. After standing for a few minutes so as to allow the cuprous oxide to settle the clear blue supernatant fluid is filtered through an asbestos mat in a weighed Gooch crucible, the precipitate collected on the mat, washed with water alcohol and ether, then dried at about 100° C., cooled and weighed.

The relative amounts of Allihn's solution and sugar solution, the length of time of heating and all other details being exactly same as recommended by Pflüger, the tables published by him<sup>1</sup> are then employed in calculating from the weight of cuprous oxide precipitate, the amount of sugar (reducing substance) present. The 85 cc. of final filtrate will of course contain variable amounts of dextrose in the different experiments. The smallest amount of blood taken in any experiment is 30 grams; assuming this blood to contain 0.1 per cent of dextrose, the 85 cc. of final filtrate will contain only 0.03 gram of dextrose. The largest amount of blood taken is 50 grams; assuming this to contain as much as 0.4 per cent of dextrose, the final filtrate will contain 0.2 gram of dextrose. The variation in percentage of dextrose in this solution is therefore 0.03-0.20 per cent which is well within the range of Pflüger's table.

---

<sup>1</sup> E. F. W. Pflüger: *Arch. f. d. ges. Physiol.*, xcvi, p. 1, 1902.

*Schenck's method.* Fifty cubic centimeters of blood are delivered into 250 cc. of a solution containing 0.8 per cent hydrochloric acid and 2 per cent mercuric chloride. Schenck originally recommended that 50 cc. of blood should first of all be delivered into 50 cc. of water, then mixed with 100 cc. of 2 per cent hydrochloric acid and then with 100 cc. of a 5 per cent solution mercuric chloride. It is, however, much more convenient to receive the blood directly into the made up reagent, it having been first of all shown that to do so does not yield results which differ from those obtained when Schenck's process is exactly followed. The mixture is well shaken and then allowed to stand for some time<sup>1</sup> until the precipitate has settled after which it is filtered without suction through a dry folded filter. Washed sulphuretted hydrogen gas is then passed through the filtrate until all the mercury is precipitated, the mercuric sulphide filtered off, an aliquot part—150 cc. (corresponding to half the blood taken)—of the filtrate removed to a flask and air bubbled through it to remove all traces of sulphuretted hydrogen, then nearly neutralized but left slightly acid, evaporated over a water bath to a small bulk, carefully neutralized, filtered and the filtrate made up to a suitable volume for the estimation of reducing power. Schenck and those who have strictly followed his directions have employed for this last purpose, the titration method of Knapp, which, however, according to Sutton is not very accurate. In a few of our experiments, Pavy's titration was employed but was not found very trustworthy for this purpose.

The chief difficulty in using Pavy's method occurs when small traces of proteid remain in the final filtrate, thus giving, with the alkaline copper solution, a biuret reaction and masking the end point of the reaction. In all the other estimations we have adopted the gravimetric method, as recommended by Pflüger, and discussed above.

To ascertain the accuracy of their respective methods, both Reid and Schenck have employed the test of adding a certain quantity of dextrose to blood and determining the amount of reducing substance (in terms of dextrose) before and after the addition of the dextrose. The difference between the two determinations should equal the amount of dextrose added. It has been found that both methods give entirely satisfactory results by this test, a fact which we have confirmed in this laboratory. Thus, by Reid's method, the percentage amount of reducing substance in a specimen of dog's defibrinated blood was found to be 0.0905. In another portion of the same blood to which 0.1005 per cent of dextrose had been added the amount

<sup>1</sup> Embden has found that prolonged standing of Schenck's reagent and blood causes some of the reducing substances to be precipitated (quoted by Liefmann and Stern, *loc. cit.*).

found was 0.191 giving a difference of 0.1002. A similar test by Schenck's method (using Pavy titration) gave a less satisfactory result, viz: to a blood containing 0.211 per cent of dextrose, 1.264 per cent dextrose was added. A sample of this contained 1.52 per cent dextrose, giving a difference of 1.310 gram. Duplicate determinations of the same sample of blood by the same method give closely agreeing results when Reid's method is employed and the same is true for Schenck's method when the gravimetric method is employed for determining the amount of reduction. The following are duplicates by Reid's method, taken at random from our protocols: 0.246 and 0.230; 0.306 and 0.312; 0.139 and 0.136; 0.223 and 0.228; 0.342 and 0.353; 0.259 and 0.260; 0.253 and 0.260; 0.155 and 0.157; 0.144 and 0.152; 0.139 and 0.121. The following are duplicates by Schenck's method: 0.196 and 0.166; 0.079 and 0.075; 0.117 and 0.123; 0.139 and 0.134; 0.123 and 0.135; 0.137 and 0.133. According to both these tests of accuracy either method is satisfactory although that of Reid gives the more constant results, but when the amount of reducing substance determined by the one method is compared with that determined by the other in the same sample of blood a very considerable discrepancy is found to exist. By Schenck's method less reducing substance is found than by Reid's. Thus by Schenck's method as above described and by Reid's method, the following amounts of reducing substance in the same sample of blood were found.

TABLE I.

REDUCING SUBSTANCE IN 100 GRAMS OF BLOOD BY		Difference.	Per cent (Reid) Difference.
Reid's method.	Schenck's method.		
gm.	gm.	gm.	
0.255	0.182	0.073	28.6
0.151	0.129	0.022	14
0.266	0.193	0.073	27
			(Pavy titration)
0.165	0.135	0.030	18
0.231	0.207	0.024	10
0.143	0.129	0.014	10

It is seen that the values obtained by Schenck's method are always much lower than those obtained by Reid's, and moreover, that the difference varies for different bloods. These results show that either one or other method is inaccurate, or that the mercuric chloride employed by Schenck precipitates certain of the reducing substances in blood which phosphotungstic acid does not.

Let us see first of all whether any errors of accuracy can be detected in either method. There are two stages at which errors are most likely to be made. The first of these is in the method of treatment of the proteid precipitate and the second, in the process employed for the estimation of the reducing power of the final filtrate.

Concerning the estimation of the reducing power of the final filtrate, we have as before stated, adopted, for both Schenck's and Reid's methods, the method of Allihn-Pflüger. In using this in the case of Reid's method, there is a possibility of too high a value being sometimes obtained on account of the presence in the final filtrate of a slight opalescence.<sup>1</sup> As far as can be seen, however, this opalescence disappears when the filtrate is mixed with Allihn's solution. To ascertain whether this opacity or some other possible impurity in the cuprous oxide precipitate does materially affect the accuracy of the methods, we have made determinations of the copper in the precipitates by the method of Volhard with the following results.<sup>2</sup>

<sup>1</sup> In Reid's process during the evaporation to small bulk of the original filtrate and washings a considerable precipitate of a white or pale blue color usually separates out. Sometimes when filtering off this precipitate, some of it passes through the filter paper with the wash water and so renders the final filtrate opalescent.

<sup>2</sup> These controls by Volhard's method were made in most cases where the results as obtained by the gravimetric process were unexpected, and the table on the following page is composed of selections from the protocols, good as well as bad results being included.

TABLE II.

Method and Number.	Amount of copper calculated from weight of $\text{Cu}_2\text{O}$ ppt.	Amount of copper found by Volhard's method.	Difference in grams.*	Per cent difference.
Reid (5).....	0.1046	0.1040	0.0006	0.6
" (2).....	0.2323	0.2397	+0.0074	+3.0
" (9).....	0.0816	0.0820	+0.0004	+0.5
" (10).....	0.1764	0.1672	0.0092	5.0
" .....	0.1098	0.1090	0.0008	0.7
" (4).....	0.1676	0.1682	+0.0006	+0.3
" (17).....	0.0735	0.0732	0.0003	0.5
" (19).....	0.0413	0.0470	+0.0057	+7.0
" (15).....	0.0675	0.0680	+0.0005	+0.6
" (29).....	0.1285	0.1308	+0.0023	+0.1
Schenck (4) .....	0.1437	0.1374	0.0063	4.0
" (3) .....	0.1600	0.1602	+0.0002	+0.15
Dextrose solution....	0.2978	0.2960	0.0018	0.6
" " .....	0.2997	0.2964	0.0033	1.1

\* In those with + sign the estimations by Volhard's method were higher than the gravimetric. Those with no sign were smaller by Volhard's than by the gravimetric method.

It will be seen that there is usually very little difference in the amount of copper found by titration as compared with that calculated from the weight of the cuprous oxide precipitates.

It is evident, therefore, that the difference in results obtained by the two methods must arise at some earlier stage in the process, viz: in the method of treatment of the proteid precipitate. In Reid's method this is thoroughly macerated with cold water and in Schenck's method it is assumed to contain the same percentage of reducing substance as the fluid and is therefore disregarded, an aliquot portion of the fluid being employed for the further stages of the process. That by treating the precipitate obtained by Reid's method, as above described, all traces of reducing substance extractable by cold water are certainly removed, we have shown by collecting several of the exhausted precipitates and again triturating them in a mortar and extracting with cold water. On evaporating the filtrate from this and heating with Allihn's solution we have been unable to detect any reduction. There is no doubt that by Reid's method



of treating the precipitate all reducing substance is removed from it.

Regarding Schenck's method, the only means by which it can be shown whether or not fluid and precipitate contain the same percentage of reducing substance are indirect, since, on account of the gummy nature of the precipitates, it is impossible to extract them with water. To test this point, Schenck added a known quantity of dextrose to defibrinated blood and found, by his method as above described, the same percentage reducing power as when an equivalent amount of dextrose was added to the proteid-free filtrate just prior to titration. It, therefore, made no difference in the results whether the dextrose was added to the blood before or after precipitation of the proteids. This seems to be the only result from which Schenck concludes that the distribution of dextrose is uniform in precipitate and solution. In two papers, published previously to that in which the above method is described, Schenck states that when the proteid of serum or of blood is coagulated by acidifying and boiling, only a portion of the added dextrose can be accounted for in the filtrate even when the coagula are most thoroughly extracted with water.<sup>1</sup> When, however, the thoroughly washed coagula are boiled with 5 per cent hydrochloric acid, reducing substance is removed from them, and indeed in almost sufficient amounts to make good the loss, when the filtrate alone is employed.

These observations led us to the next step, viz: to see whether by boiling the blood with Schenck's reagent reducing substances would become dislodged from the precipitate and appear in the filtrate, thus bringing the results up to the same level as those obtained by Reid's method. In doing this, we have as before compared the percentage reducing power of the same blood by Reid's method with that found by using Schenck's method combined with heat.<sup>2</sup>

<sup>1</sup> Fr. Schenck: *Arch. f. d. ges. Physiol.*, lv, p. 203, 1894; xlvii, p. 621, 1890.

<sup>2</sup> In employing heat it was necessary to weigh the solution before boiling and make up to this weight after boiling. An aliquot portion of the HgS-free filtrate was then employed for the further stages of the process.

ble gives the results:

TABLE III.

Schenck.	Difference.	Per cent difference.
0.1397	0.055	28.7
0.1345		
0.177	0.037	17.2
0.181	0.078	30
0.193	0.063	24.2
0.120	0.054	31
0.077	0.037	32.4

that boiling does not increase the amount of dextrose as found by Schenck's method. Since the method gives satisfactory results when tested regarding the addition of a known amount of dextrose to blood, Schenck's method must be due to the reaction of the reagent with the proteids some of the reducing substances which phosphotungstic acid does not precipitate. It cannot be dextrose for the reason just stated, therefore be some other reducing body such as glycerol or pentoses. Before considering this possibility it should be pointed out that Embden has found that the reaction of the mercuric chloride with blood to cause dextrose to be precipitated. Liefmann and Stern<sup>1</sup> found that the mixture of blood and reagent to stand six hours but Embden himself in a previous research waited 15 minutes.<sup>2</sup>

The amount of glycuronic acid in blood (of the ox) has been found by Paul Meyer.<sup>3</sup> The same author has found that the amount of dog's blood, unlike that of horse's blood, contains pentose, but that not more than 2.5 to 5 per cent of the reducing power of dog's blood can be accounted for by the pentose substance. The amount of dextrose thus coming from the diet. The presence of pentose is uncertain.

<sup>1</sup> Stern: *Biochem. Zeitschr.*, i, p. 297, 1906.

<sup>2</sup> Liefmann and Stern: *loc. cit.*, p. 300.

<sup>3</sup> *Zeitschr. f. physiol. Chem.*, xxxii, p. 518, 1901.